Utrophin A upregulation by FDA-approved drugs for the treatment of Duchenne Muscular Dystrophy

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Authorizations

**Chapter 2:**

**Chapter 3:**

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Abstract

Duchenne muscular Dystrophy (DMD) is a disorder caused by mutations in the dystrophin gene, preventing the production of the functional dystrophin protein which assures maintenance of the myofiber integrity throughout muscle contraction. A lack of dystrophin results in severe muscle degeneration and regeneration accompanied by a loss of muscle function. Many pre-clinical and clinical studies are focused on developing strategies to counteract the detrimental effects of DMD; however, there is no cure. One such approach consists of upregulating the endogenous protein utrophin A in dystrophic muscle, which, once highly expressed at the sarcolemma, could functionally compensate for the lack of dystrophin. Recent evidence demonstrates that utrophin A expression is regulated at its 3’ and 5’UTR through post-transcriptional and translational events. Therefore, in the work presented here, we hypothesized that repurposing FDA-approved drugs that target the signaling pathways involved in post-transcriptional and translational regulation of utrophin A will be an efficient approach in rapidly bringing new therapeutic interventions for DMD.

In this work, we repurposed four promising FDA-approved drugs able to stimulate utrophin A expression levels in dystrophic muscles: the anti-coagulant drug Heparin, the anti-inflammatory drug Celecoxib, the β-adrenergic receptor blocking agent Betaxolol and the cholesterol-lowering drug Pravastatin. These drugs induce significant improvements in the dystrophic phenotype of mdx mice. This includes amelioration of muscle fiber integrity and muscle function as well as promoting morphological and fiber type changes in mdx mice muscles. Collectively, this thesis describes the potential of a repurposing approach to activate key post-transcriptional and translational pathways involved in utrophin A’s regulation in the hopes of developing new therapeutics for the treatment of DMD.
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Abbreviations

4E-BP- eIF4E-binding protein
6MWD T- 6-minute walking distance test
AChE- acetylcholinesterase
AAV- adeno-associated viruses
AICAR- 5-amino-4-imidazolecarboxamide riboside
AMPK- 5’ adenosine monophosphate-activated protein kinase
AON- antisense oligonucleotide
AP-1- activator protein-1
ARE- AU-rich elements
AREMD- ARE-mediated decay
ATP- adenosine triphosphate
AUBP- ARE-binding proteins
BMD- Becker Muscular Dystrophy
Ca^{2+}- calcium
Cas9- CRISPR-associated protein 9
Cel- celecoxib
CN- calcineurin
CoQ10- coenzyme Q10
COX-2- cyclo-oxygenase 2
CRISPR- clustered regularly interspaced short palindromic repeats
CSA- cross-sectional area
CTGF- anti-connective tissue growth factor
CVB3- coxsackievirus B3

DAPC- dystrophin-associated protein complex

DMD- Duchenne Muscular Dystrophy

DIA- diaphragm

Dusp10- dual specificity phosphatase-10

E2F1- E2F transcription factor 1

ECM- extracellular matrix

EDL- extensor digitorum longus

eEF1A- elongation factor 1A

eIF- eukaryotic initiation factor

ER- endoplasmic reticulum

ERF- ets-2 repressor factor

ERK- extracellular signal-related kinase

FDA- food and drug administration

FGF1- fibroblast growth factor 1

Fnip1- null-folliculin interacting protein-1

GABP- growth-associated binding protein

GRMD- golden retriever model of DMD

GRSF- G-rich RNA sequence binding factor 1

HCV- hepatitis C virus

HDAC- histone deacetylases

HDR- homology-directed repair

HER- heregulin to its receptor
HR- hairless
HSA- human skeletal α-actin
HuR- human antigen-related protein
IGF-IR- insulin-like growth factor receptor Type I
IgM- immunoglobulin M
IRES- internal ribosomal entry site
ITAF- IRES trans-acting factors
KH- K-homology
KSRP- K-homology splicing regulatory protein
LA- autoantigen
LTBP4- latent TGF-β-binding protein 4
m7G- 7-methylguanylate cap
mdx- X-linked muscular dystrophy
MEF2- myocyte enhancer factor-2
MeSC- mesenchymal stem cells
MHC- myosin heavy chain
miRNA- microRNA
MRF- myogenic regulatory factors
MSC- mechanosensitive ion channels
MARK2- serine/threonine-protein kinase
Myd88- myeloid differentiation primary response gene 88
MyoD- myogenic differentiation factor D
NEFA- non-esterified fatty acid
NFAT - nuclear factor of activated T-cells
NHEJ - non-homologous end-joining
NMJ - neuromuscular junction
NO - nitric oxide
NOS - NO synthase
nNOS - neuronal nitric oxide synthase
NSAID - nonsteroidal anti-inflammatory drug
\( \text{O}_2^- \) - superoxide
OXPHOS - oxidative phosphorylation
p38 MAPK - p38 mitogen-activated protein kinases
PABP - poly(A) binding protein
PAR-1b - polarity-regulating kinase partitioning-defective 1b
Pard3 - partitioning-defective 3
PARN - poly(A) specific ribonuclease
PCBP2 - poly(rC) binding protein-2
PGC-1\( \alpha \) - peroxisome proliferator-activated receptor \( \gamma \) coactivator 1\( \alpha \)
PMO - phosphorodiamidate morpholino oligomers
PPAR\( \beta/\delta \) - peroxisome proliferator-activated receptor-\( \beta/\delta \)
PSF - polypyrimidine tract binding protein-associated splicing factor
PTB - polypyrimidine tract binding protein
RhoA - ras homolog gene family, member A
ROS - reactive oxygen species
RYR - ryanodine receptor
SAC- stretch-activated calcium channels
SIRT- sirtuin
SMA- Spinal Muscular Atrophy
SOCE- store-operated Ca\(^{2+}\) entry
SR- sarcoplasmic reticulum
SREBP-1- sterol-regulatory-element-binding protein 1
STIM1- stromal interaction molecule 1
TA- tibialis anterior
tcDNA- tricyclo-DNA
TGF-β- transforming growth factor beta
TLR- toll-like receptor
TMEV- Theiler's murine encephalomyelitis virus
tRNA- transfer RNA
TRPC- transient receptor potential canonical channels
TRPV2- TRP vanilloid 2
TTP- Tristetraprolin
Unr- Nras
UTR- untranslated region
UTR-A - utrophin A
VC- variance coefficient
YB1- Y-box binding protein 1
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Chapter 1 – General introduction
1.0 Introduction

Skeletal muscle comprises nearly 40% of the total human body mass and is the largest tissue in the body. Muscles play a vital role to generate body movement, to maintain posture, it enables breathing, feeding, proper vision and strongly contributes to the regulation of metabolism and hormonal stability. Thus, many disorders that affect proper function of muscle are detrimental and life threatening. Although there are symptom-relieving interventions for patients suffering with muscular diseases such as muscular dystrophy, there are no effective cures. This is why it is imperative to find novel treatments that could alleviate the devastating features of muscular dystrophies. In the series of studies presented here, we will discuss our most recent findings focused towards pharmacological stimulation of the endogenous protein called utrophin A, in skeletal muscles, as a therapeutic approach for the treatment of Duchenne Muscular Dystrophy.

1.1 Duchenne Muscular Dystrophy: A Severe Neuromuscular Disorder

Duchenne Muscular Dystrophy (DMD) is one of the most common inherited paediatric neuromuscular diseases. In fact, this disorder accounts for over 80% of cases of muscular dystrophies around the world (Emery, 1991). Birth prevalence of DMD has been shown to range from 15.9 to 19.5 per 100,000 male births in the United States and the United Kingdom (Ryder et al., 2017). These patients suffer from severe muscle degeneration which results in muscle weakness, respiratory impairment and cardiomyopathy. Loss of ambulation arises at approximately 12 years of age and progresses until the age 16–18, after which ambulation is completely lost (Mayer et al., 2015). Ventilation usually starts at the age of 20 (Mayer et al., 2015; Ryder et al., 2017) and death occurs due to respiratory or cardiac complications when patients are in their late 20s (Bach et al., 1987; Guiraud et al., 2015a).
DMD is a disorder caused by mutations in the *DMD* gene on the Xp21.1 chromosome. The *DMD* gene is considered as one of the largest known human genes which contains 79 exons, encodes for a 14-kb mRNA and a 427kDa protein (Hoffman et al., 1987). Approximately 65%-70% of patients who suffer from DMD have either a single-exon deletion, multi-exon deletion or a duplication in the dystrophin gene and tend to be found in two hot spot areas near the N-terminus (exons 3-7) and within the central rod domain (exon 45-55) (Flanigan et al., 2009; Lee et al., 2012; Traverso et al., 2006). The remaining 20-30% have small deletions, small duplications or point mutations (nonsense or missense) (Ankala et al., 2015; Bladen et al., 2015; Bushby et al., 2010). Patients that carry in-frame as opposed to out-of-frame mutations in DMD usually present with a less severe disorder called Becker Muscular Dystrophy (BMD) (Monaco et al., 1988). Overall, this demonstrates the complexity and severity of DMD and why finding a treatment is arduous.

1.2 Dystrophin and the Dystrophin-Associated Glycoprotein Complex (DAPC)

The dystrophin protein is composed of four main domains which includes: an N-terminal calponin homology module containing an actin-binding domain, a central rod domain that consists of 24 spectrin-like triple helical coiled coils, a cysteine-rich domain, and a C-terminal domain (Hoffman et al., 1987; Koenig and Kunkel, 1990) (Figure 1). The central rod domain makes the protein more flexible (Hoffman et al., 1987; Koenig and Kunkel, 1990). Dystrophin binds the dystrophin-associated protein complex (DAPC) at the muscle membrane and the actin cytoskeleton which provides muscle membrane stability (Ervasti et al., 1990).

The DAPC is a complex containing membrane-associated proteins that is necessary for skeletal muscle fiber integrity. This complex contains dystrophin, α and β-dystroglycans, α, β, γ and δ sarcoglycans, sarcospan, α1, β1, β2; γ1 and γ2 syntrophins as well as α-dystrobrevin (Adams
et al., 1993; Bönnemann et al., 1995; Campbell and Kahl, 1989; Croesbe et al., 1997; Ervasti et al., 1990; Grady et al., 1999; Ibraghimov-Beskrovnaya et al., 1992; Lim et al., 1995; Nigro et al., 1996; Noguchi et al., 1995; Piluso et al., 2000; Yang et al., 1995). When interacting with the DAPC, dystrophin binds the transmembrane protein β-dystroglycan. The extracellular domain of β-dystroglycan binds α-dystroglycan which binds laminin-2 in the basal lamina (Ervasti and Campbell, 1991, 1993). Overall, the DAPC serves as a link between the extracellular matrix (ECM) and the sarcolemmal cytoskeleton of the cell membrane to protect muscle cells from contraction-induced damage (Weller et al., 1990) (Figure 1). In fact, mutations found in genes encoding dystrophin, all four sarcoglycans and laminin-2 are responsible for a variety of muscular dystrophies including DMD/BMD, limb–girdle muscular dystrophy type 2C-F and congenital muscular dystrophy (Allamand et al., 1997; Bönnemann et al., 1995; Hoffman et al., 1987; Lim et al., 1995; Nigro et al., 1996; Noguchi et al., 1995; Roberds et al., 1994; Yang et al., 1995). Dystrophin also binds neuronal nitric oxide synthase (nNOS) and permits its localization to the sarcolemma where it functions to modulate vasodilation of blood vessels during muscle contraction (Chang et al., 1996; Percival et al., 2008, 2010; Thomas et al., 1998, 2003). However, the role of the DAPC and dystrophin goes beyond structural support. Notably, they are involved in cell signaling such as cell survival and cellular defense mechanisms but also they regulate the epigenetic activation of muscle stem cell commitment (Chang et al., 2018; Dumont et al., 2015; Rando, 2001). Dystrophin is critical for proper function of muscles, which is why studies are focused on restoring its expression or upregulating other molecules that compensate for its absence.
Figure 1. Dystrophin association with members of the DAPC at the muscle fiber sarcolemma.

Dystrophin and the DAPC forms at the sarcolemma of muscle fibers to provide a link between the ECM and the intracellular cytoskeleton to protect the muscle from contractions. The N-terminal domain (N) of dystrophin and specific spectrin repeats interacts with F-actin filaments. Dystrophin has a rod domain, made flexible by four hinge regions (H1, H2, H3, and H4) and a cysteine-rich region (CR) that binds β-dystroglycan. Dystrophin associates with α-dystrobrevin and syntrophins α and β at its C-terminal domain (C). The extracellular domain of β-dystroglycan interacts with α-dystroglycan which binds laminin-2 at the ECM. The DAPC is also supported at the sarcolemma by the binding of the sarcoglycans (α, β, δ and γ) and sarcospan. The syntrophins recruit nNOS at the muscle fiber membrane with their PDZ domains to mediate blood flow to the muscle. Furthermore, dystrophin can also interact with nNOS at its spectrin repeats 16/17.
1.3 DMD Animal Models

Preclinical animal testing has played an important role in assessing safety and efficacy of either drugs or therapeutic approaches envisioned for therapy in humans. In addition, animal models have been vital for basic discovery and to decipher mechanisms involved in diseases. The Food and Drug Administration (FDA) agency requests acute, subacute and chronic dosing studies in a rodent and a second species before moving to clinical trials (Andrade et al., 2016). DMD models including rodents, canines, felines and zebrafish have been used over the years to study this disorder, however, the mdx mice and the golden retriever DMD model are the most commonly used (Collins and Morgan, 2003; Kawahara and Kunkel, 2013; McGreevy et al., 2015).

1.3.1 The DMD Mouse Models

The C57BL/10ScSn-\textit{Dmd}^{mdx}/J, mdx mouse is the most commonly used mouse model for DMD. This mouse model has a premature stop codon mutation within exon 23 of the \textit{DMD} gene, resulting in the absence of dystrophin (Bulfield et al., 1984; Ryder-Cook et al., 1988; Sicinski et al., 1989). The mdx mouse was first identified because of its impaired muscle pathology and raised serum creatine kinase levels (an enzyme released in the serum from damaged muscle) (Bulfield et al., 1984). With an onset of the dystrophic pathology at 3 weeks of age, the mutants experience mild clinical symptoms: muscle fibers undergo cycles of necrosis and regeneration along with immune cell infiltration (McGeachie et al., 1993). The dystrophic pathology develops slowly in the mdx mouse model with a substantial accumulation of connective tissue within skeletal muscle after ~15 months of age. (Lefaucheur and Sebille, 1995). The mdx mouse also has increased muscle mass compared to the wild-type mouse which is attributed to the ongoing degeneration and regeneration cycles and extensive hypertrophy (Dupont-Versteegden and McCarter, 1992; Lynch
et al., 2001; Pastoret and Sebille, 1993). The mdx mouse model remain the most widely used DMD model for preclinical research for DMD. The mdx mouse is commercially available, easy to breed and has a long-life expectancy (Chamberlain et al., 2007). Modifications to the original mdx mouse model have expanded the available mouse models to study DMD. These include: $\text{mdx}^{\text{cv-5cv}}$ (with several point mutations which lead to the loss of dystrophin protein), $\text{mdx52}$ (deletion of exon 52 which also leads to the loss of dystrophin protein) and DMD-null mouse (deletion of the entire dystrophin gene). $\text{mdx}^{\text{cv-5cv}}$ and the $\text{mdx52}$ features similarly to the original mdx mouse, while DMD-null mouse models show a more severe phenotype together with increased muscle hypertrophy and necrosis (Gawlik, 2018).

Distinct mdx double mutant mice displaying enhanced dystrophic pathologies have also been used as more severe models of DMD. For instance, the double knockout dystrophin/utrophin mouse model. Utrophin was originally described as dystrophin-like because of its similar homology (Deconinck et al., 1997). Along with the muscle weakness and necrosis already seen in the original mdx mouse model, this double knockout shows signs of kyphosis, tip toe walking, breathing disabilities by 6 weeks and early onset cardiac failure (Deconinck et al., 1997; Janssen et al., 2005). By week 10, double knockout mice, show morphological muscle defects, severe inflammation, fibrosis and a dramatically reduced life span (Deconinck et al., 1997). Another double knockout mutant mdx mouse is the $\text{mdx}/\text{MyoD}$ model. Myogenic differentiation factor D (MyoD) plays an important role in skeletal muscle differentiation and regeneration, thus, reducing its expression in combination with the loss of dystrophin results in a severe myopathy (Megeney et al., 1999). Even though double knockout mice models elicit a more severe phenotype which are more consistent with symptoms found in human patients, the breeding complications and reduced life span of these mice make them difficult to manage for DMD research.
1.3.2 The Golden Retriever Model of Duchenne Muscular Dystrophy (GRMD)

Sporadic cases of canine purebreds affected by dystrophinopathies have been reported over the years such as in the Golden Retriever (GRMD) (Sharp et al., 1992), Rottweiler (Shelton and Engvall, 2005), German Short-Hair Pointer (Schatzberg et al., 1999) and Miniature Poodles (Sánchez et al., 2018). However, the GRMD is the most commonly used canine model for DMD. Symptoms elicited in GRMDs are closer to human DMD patients, thus making it a more relevant model. These dogs suffer from early onset muscle degeneration, followed by necrosis from birth and have highly increased creatine kinase levels at as early as 1-2 days old (Shelton and Engvall, 2005; Valentine et al., 1989). Respiratory and cardiac failure also starts at a young age and they develop severe muscle fibrosis at approximately 6 months. Another feature observed in GRMD is the enlargement of the base of the tongue which is also seen in human patients with DMD due to muscle fiber hypertrophy (Shelton and Engvall, 2005; Valentine et al., 1989). Even though the GRMD model is more clinically relevant than the mdx mouse, usage has been limited due to space requirements, higher expenses and availability of dogs. Thus, the mdx model is still the preferred choice for preclinical research.

1.4 Current Supportive Therapy for DMD Patients

A multidisciplinary standard of care protocol is followed to manage the multiple complications faced by DMD patients. This includes ambulatory, respiratory, cardiac, gastrointestinal, nutritional and bone health management to name a few (Birmkrant et al., 2018a, 2018b, 2018c). The use of glucocorticoid therapy is the main intervention used for the treatment
of DMD (Birnkrant et al., 2018a). The benefits of glucocorticoids consist of slowing the loss of ambulation, maintenance of upper limb and respiratory function, and decreased risk of scoliosis surgery (Lebel et al., 2013). The fact that glucocorticoids continue to represent the standard of care for DMD patients provides strong evidence for the challenges surrounding the implementation of other treatments including genetic, cellular and pharmacological therapies. However, there is a total of 214 promising studies, diagnostic tests and treatment trials in various stages of enrollment (the NIH Clinical Trials.gov) in hopes to find a treatment for DMD. These studies can be separated in two main groups: 1) therapies that aim to restore dystrophin expression in dystrophic muscles and 2) therapeutic approaches based on improving disease associated outcomes through alternative pathways.

1.5. Restoration of the Dystrophin Protein in Dystrophic Muscles

Mutations in the gene encoding dystrophin is the primary cause of DMD disorder and results in a severe ongoing degeneration of the muscle fibers (Flanigan et al., 2009; Hoffman et al., 1987; Lee et al., 2012; Mayer et al., 2015; Traverso et al., 2006). Studies have focused their efforts at the source of the problem, by either attempting to deliver dystrophin to the muscles, or to re-establish the proper reading frame of the dystrophin gene to restore the functional dystrophin protein altogether. These techniques include cell therapy, gene therapy, exon skipping, suppression of premature stop codons and gene editing.

1.5.1 Cell Therapy to Restore Damaged Muscles

Skeletal muscle has a great stem cell-dependent regenerating ability. In fact, adult stem cells are activated in response to muscle injury to regenerate damaged muscle tissue. Muscle
satellite cells (muscle-derived stem cells), can achieve either symmetric or asymmetric division (Kuang et al., 2007, 2008). Symmetric division creates two self-renewing daughter cells to maintain the pool of quiescent stem cells, or two committed differentiating daughters. Asymmetric cell division generates a committed myoblast (myogenic precursor cell) ready for expansion and a self-renewing daughter cell (Kuang et al., 2007; Le Grand et al., 2009; Rocheteau et al., 2012). Myogenic precursor cells will undergo many rounds of division prior to differentiation and then will fuse with the damaged myofiber to repair the damaged muscle and to restore its integrity (Kuang et al., 2007, 2008; Le Grand et al., 2009; Rocheteau et al., 2012). Regenerative medicine using stem cells has been an attractive method explored for the treatment of muscular dystrophies (Crist, 2017; Danisovic et al., 2018). A variety of cell types have shown beneficial myogenic effects in dystrophic animal models, such as muscle satellite cells, muscle-derived stem cells as well as cells derived from bone marrow and vessel walls (Benedetti et al., 2013).

A phase I/II clinical trial, in 2015, consisted of intra-arterial transplantation of mesoangioblasts (vessel-associated stem cells isolated from the skeletal muscle vasculature) in five DMD patients (Cossu et al., 2015). This was based on a promising preclinical outcome where GRMD dogs subjected to a mesoangioblasts transplant, elicited improved muscle function and dystrophin protein recovery (Sampaolesi et al., 2006). Yet, the results from the clinical trial revealed low level of donor DNA, little to no effect on ambulation and no functional improvements. Similar results were found in the analysis of a bone marrow-transplanted 1-year-old DMD patient, who showed little but detectable amounts of donor-derived skeletal muscle cells 13 years post transplant (Gussoni et al., 2002). This lack of efficiency is usually due to difficulties in delivery, engraftment and cell survival (Cossu et al., 2015; Gawlik, 2018). A new phase I/II clinical trial to test the beneficial effects of Wharton jelly-derived mesenchymal stem cells (MeSC)
in DMD patients is in progress (Dai et al., 2018). Wharton jelly-derived MeSCs have been shown to have the capacity to suppress apoptosis of muscle cells, which in part, could help improve the efficiency of cell therapy (Dai et al., 2018). Another approach is using human induced pluripotent stem cells (hiPSC), derived from DMD patient fibroblasts, and subjecting them to exon skipping or gene editing, to restore dystrophin in DMD muscles following engraftment of the corrected hiPSC cells (Li et al., 2015; Young et al., 2016).

### 1.5.2 Gene Therapy: Delivering Dystrophin to Dystrophic Muscles

Gene delivery to specific tissues has been studied thoroughly since it was first developed in the 1970s (Friedmann and Roblin, 1972; Gregorevic et al., 2004), as a therapeutic approach for the treatment of diseases. Virus-driven delivery of genes to dystrophic muscles, in attempts to improve DMD conditions and restore dystrophin expression, has been successful in many animal models (Harper et al., 2002; Kodippili et al., 2018; Wang et al., 2012; Wasala et al., 2018). The most promising gene delivery system are Adeno-associated viruses (AAV) that are used to introduce either a miniaturized dystrophin gene (mini or micro-dystrophin) (Harper et al., 2002) or other substitute genes that can compensate for the absence of dystrophin (described below). Dystrophin delivery with AAV is complex since full-length dystrophin is too large for the capacity of the AAV vectors (Harper et al., 2002). Accordingly, scientists have generated functional miniaturized versions of dystrophin by deleting multiple regions (notably parts of the central rod domain and carboxy-terminal domain) of the protein (Harper et al., 2002). This gene therapy approach has been shown to reverse many of the dystrophic features (Harper et al., 2002; Kodippili et al., 2018; Wang et al., 2012; Wasala et al., 2018). However, the biggest set backs of this technique are the many injections that need to be administered to the patients to insert the vectors.
in their muscles and the host immune responses to either the AAV capsid proteins and/or the transgene product (Mendell et al., 2010; Okada and Takeda, 2013; Wang et al., 2011b, 2012; Yue et al., 2008). Thus, combinatorial therapy with immunosuppressants (Chicoine et al., 2014; Ohshima et al., 2009; Wang et al., 2012) and testing novel AAV constructs such as muscle-specific gene regulatory cassettes (Himeda et al., 2011) are being explored. Promising results from a 90-day phase I/IIa clinical trial with AAVrh74.MHCK7.micro-Dystrophin treatment (designed to maintain spectrin-like repeats 2 and 3) from Sarepta Therapeutics were recently announced and demonstrated good micro-dystrophin expression in muscles of three patients with DMD. These patients also had reduced creatine kinase and did not show serious adverse events except for an increase in gamma-glutamyl transferase, which was controlled with an increased steroid dose (Sarepta Therapeutics, 2018). Thus, although the method of reintroducing dystrophin in dystrophic muscles requires additional work to improve tissue uptake and unwanted immune responses, this promises to be a successful treatment for DMD patients.

1.5.3 Antisense Oligonucleotide Technology to Induce Exon Skipping in the \textit{DMD} Gene

Learning the range and the location of mutations in the \textit{DMD} gene has became crucial for therapies focused on re-establishing the reading frame with antisense oligonucleotide (AON) technology. AAVs can be used to transfer AON cassettes to promote pre-mRNA exon-skipping in dystrophic muscles and restore the expression of a truncated but functional dystrophin protein (Barbash et al., 2013; Bish et al., 2012; Yokota et al., 2009). Alternatively, synthesized AONs can also be delivered into tissues using a variety of chemical backbones including peptide nucleic acids, 2’O-methyl-phosphorothioate oligoribonucleotide or phosphorodiamidate morpholino oligomers (PMO). AONs specific for exon 51 skipping reached clinical trials, notably Drisapersen.
(PROO51) (PMO), but failed at clinical trial phase III due to insignificant improvement in the 6 Minute Walking Distance Test (6MWDT) (Lu et al., 2014). These inconsistencies are usually due to low efficiency of AONs in some tissues as they are unable to penetrate the bilipid layer (Goyenvalle et al., 2015; Lu et al., 2014). Furthermore, increasing the dose of AONs resulted in kidney toxicity (Lu et al., 2014). Modified versions of the chemical backbones of AONs, such as the new class of AONs made of tricyclo-DNA (tcDNA oligomers), have been explored in order to increase tissue uptake and permit AONs to enter the heart (Goyenvalle et al., 2015). Recent success with the drug Exondys51 (Eteplirsen), used to skip exon 51 in the DMD gene, has been conditionally approved by the FDA (Irwin and Herink, 2017; Niks and Aartsma-Rus, 2017). Clinical trial results from a long-term assessment of patients treated weekly with Eteplirsen, displayed the expected skipping of exon 51 after 180 weeks (Charleston et al., 2018). Despite these promising new results, exon skipping is not suitable for all mutations and is only applicable for a small percentage of DMD patients.

### 1.5.4 Suppression of Premature Stop Codons

A small majority (approximately 13-15%) of DMD patients have non-sense mutations (Malik et al., 2010) causing a premature stop codon within the coding region, thus creating a short non-functional dystrophin protein. Accordingly, a read-through approach was developed using small molecules, such as aminoglycosides, that affects the conformation of dystrophin encoding mRNA, permitting ribosomal subunits to read through a premature stop codon (Baradaran-Heravi et al., 2016).

Interestingly the aminoglycoside antibiotic, Gentamicin, was revealed to induce read-through of premature stop codons in the DMD cell lines resulting in the production of full-length
functional dystrophin protein (Baradaran-Heravi et al., 2017; Malik et al., 2010, 2010). Furthermore, Gentamicin-treated mdx mice increased dystrophin’s sarcolemmal localization and muscle contraction performance. Clinical trials with DMD and cystic fibrosis patients have shown that therapeutically relevant levels of read-through is not possible within the non-toxic dosage for gentamicin (Clancy et al., 2001; Malik et al., 2010), thus new molecules were explored. One such promising read-through agent is Ataluren (PTC124/ PTC Therapeutics) also known as Translarna. A 2–8 week treatment of primary human muscle cells and mdx mice with this small molecule elicited the dystrophin expression in affected tissues and rescued muscle function (Welch et al., 2007). Ataluren moved forward into clinical trials and has been well tolerated by most DMD patients. Phase III clinical trial results reveal that this drug has significant effects on the 6MWD of groups that walk between 300m to 400m (McDonald et al., 2017). An additional trial (NCT03179631) to study the long-term efficacy and safety of Ataluren is currently in progress. Although the approach to suppress premature stop codons in the dystrophin gene, with agents such as Ataluren, is moving along nicely through clinical trials, it is only applicable to approximately 10% of the DMD patient population due to the inconsistency in mutations from one patient to another (Guiraud et al., 2015a).

1.5.5 Gene Editing to Restore Dystrophin Expression

Gene editing using clustered regularly interspaced short palindromic repeats (CRISPR) technology has been recently adapted for gene correction in the context of DMD (Amoasii et al., 2018; Bengtsson et al., 2017; Duchêne et al., 2018; Hakim et al., 2018; Ifuku et al., 2018; Long et al., 2014; Nelson et al., 2016; Xu et al., 2018). When combining CRISPR with Cas9 (CRISPR-associated protein 9) enzyme and by targeting specific mutated areas of the DMD gene for editing,
splicing sequences can be altered or disrupted to skip exons to promote the production of the dystrophin protein. Briefly, Cas9 is guided by a guide-RNA to bind to a specific gene locus in order to induce a double-strand break in the \textit{DMD} gene. This break is then repaired either by non-homologous end-joining (NHEJ), which results in an insertion or deletion to restore the proper reading frame, or by homology-directed repair (HDR) with an exogenous template to precisely correct the target gene (Doudna and Charpentier, 2014; Long et al., 2014).

CRISPR/Cas9–mediated genomic editing has now been shown in several studies \textit{in vitro} and \textit{in vivo}, to be able to correct the mutation responsible for DMD in DMD patient muscle cells (Duchêne et al., 2018), stem cells (Ifuku et al., 2018; Tabebordbar et al., 2016) mdx mice (Bengtsson et al., 2017; Hakim et al., 2018; Long et al., 2014; Nelson et al., 2016; Xu et al., 2016) and other DMD animal models (Amoasii et al., 2018; Chen et al., 2015; Nakamura et al., 2014). More importantly, this approach results in improvement of skeletal muscle activity and has overcome the difficulties of rescuing the cardiac function (Hakim et al., 2018; Long et al., 2014; Nelson et al., 2016; Xu et al., 2016, 2018). There are several potential avenues to apply this technology to DMD patients. Once such delivery method of CRISPR/Cas9 would be to perform genomic editing within the germ line of humans, although, this is not ethically accepted. An alternative method is via AAV (Hakim et al., 2018) delivery; however, this also has many pitfalls (as described above). Editing of satellite cells and DMD patient-derived induced pluripotent stem cells, by the CRISPR/Cas9 system, represents a promising alternate approach to promote muscle repair in dystrophic muscles (Ifuku et al., 2018; Tabebordbar et al., 2016). In fact, Dumont \textit{et al.} describes a new role of dystrophin in satellite cells to regulate their polarity and asymmetric division through interaction with Serine/threonine-protein kinase (Mark2) and regulation of Partitioning-defective 3 (Pard3) polymerization (Dumont et al., 2015). Hence, correcting
dystrophin in stem cells might be important for proper muscle repair in DMD. However, CRISPR/Cas9 has been linked to the promotion of off-target events and induction of an immune response (Lin and Wong, 2018), thus, further research is required.

1.6 Therapies Based on Improving Disease Associated Outcomes Through Alternative Pathways.

The absence of dystrophin causes a devastating domino of secondary symptoms which strongly increases the severity of the DMD disorder. These pathologies cause a chain-reaction of detrimental symptoms in DMD patients. Due to the membrane instability and defective regeneration of myofibers, extracellular calcium influx is permitted to enter the cytoplasm which can activate the NF-kB inflammatory pathway (Miyatake et al., 2016; Schaecher et al., 2004). Atypical increase of inflammatory macrophages arises in the muscle, provoke chronic inflammation, necrosis and replacement of the muscle by connective tissue (Miyatake et al., 2016). Consequently, research focused on offsetting inflammation, calcium (Ca$^{2+}$) influx, oxidative stress and fibrosis is a promising approach to improve the dystrophic phenotype (Miyatake et al., 2016; Shieh, 2018). In fact, a clinical trial is presently recruiting DMD patients to study endomysial fibrosis, deregulated inflammation response and calcium influx dysfunction in order to better understand the relationships between each secondary pathology in dystrophin-deficient humans (Clinical Trials.gov, trial NCT01823783). The many potential treatments targeting signaling pathways aimed at improving functional outcome of dystrophic muscles include: therapies targeting endogenous genes to restructure the DAPC as well as anti-inflammatory, calcium dysregulation, anti-fibrotic and oxidative stress treatments which will be described below.
1.6.1 Anti-Inflammatory Treatment in Dystrophic Muscles

The skeletal muscle is a secretory organ that releases cytokines (also called myokines) to mediate communication between the muscle and other organs in the endocrine system thus playing an important role in the inflammatory process (Miyatake et al., 2016). Healthy muscles endure consistent mechanical injury, followed by muscle degeneration and activation of acute inflammation. Moreover, muscle regeneration is driven by satellite cells to restore the muscles (Yang and Hu, 2018). The inflammation response is thought to play an essential role in bridging initial muscle injury responses and timely muscle injury repair. However, in DMD, due to the many cycles of degeneration and regeneration, chronic inflammation is increased significantly and is damaging to the muscle. NF-κB, one of the major transcription factors regulating inflammatory pathways, is an important target for anti-inflammatory therapy.

As mentioned above, glucocorticoid therapy is the main intervention used for the treatment of DMD. Prednisone (0.75 mg/kg/day) and deflazacort (0.9 mg/kg/day) are the most commonly prescribed glucocorticoids for DMD (Birnkrant et al., 2018a). Glucocorticoids have anti-inflammatory effects, in part, by suppressing NF-κB and activator protein-1 (AP-1), which inhibits coactivator molecules responsible for acetylating histones involved in switching on transcription of inflammatory genes (Barnes, 2006). Glucocorticoids also improve muscle strength in DMD patients, however, the molecular pathways controlling the beneficial versus detrimental side effects are not yet understood (Bonifati et al., 2000; Escolar et al., 2011; Moxley et al., 2010). A double-blind trial comparing benefit-to-risk relation for both deflazacort and prednisone is presently ongoing (Guglieri et al., 2017).

Recently, a study demonstrated the beneficial effects of another anti-inflammatory drug called Vamorolone (VBP15), on the dystrophic phenotype of mdx mice independently of
undesired hormonal, growth, or immunosuppressive effects often promoted by glucocorticoid treatment (Heier et al., 2013). This indicates that alternate anti-inflammatory drugs should be further investigated for DMD. As an alternative, nonsteroidal anti-inflammatory drugs (NSAIDs) were studied in DMD models. NSAIDs are cyclo-oxygenase (COX) enzyme inhibitors. These enzymes induce production of prostaglandins and lipid autacoids from arachidonic acid, leading to an inflammatory response (Ricciotti and FitzGerald, 2011). Mdx mice treated for 8-11 weeks with three types of NSAIDs were compared: non-specific COX inhibitors (aspirin and ibuprofen) and one specific COX-2 inhibitor (parecoxib). Results show that the NSAIDs improved inflammation, fiber necrosis, muscle morphology and aspirin in particular ameliorated resistance to muscle fatigue (Serra et al., 2012a).

1.6.2 Targeting Calcium Dysregulation

Deregulation of Ca\(^{2+}\) homeostasis in damaged muscles lacking dystrophin is mediated by three main components; first, the presence of leaky Ca\(^{2+}\) release channels such as mechanosensitive ion channels (MSC) and stretch-activated calcium channels (SAC) [for example: the transient receptor potential canonical channels (TRPC)] (Fong et al., 1990; Hopf et al., 1996; Matsumura et al., 2011). Researchers have found that the mechanosensitive cationic TRP vanilloid 2 (TRPV2) channel, which are typically located in intracellular membrane compartments, are amplified at the plasma membrane of dystrophic muscle fibers (Iwata et al., 2003, 2009). Depletion of TRPV2 in mdx mouse muscles reduce necrosis and regeneration, thus this presents an important pathway for therapy (Iwata et al., 2009). Interestingly, small molecules targeting MSCs are being explored for the treatment of DMD. GsMTx4, a non-selective new class of mechanosensitive Ca\(^{2+}\) channel-blocking peptides, have been shown to reduce sarcolemma Ca\(^{2+}\) influx and decrease predisposition
to contraction induced injury in various mdx mouse models (Suchyna and Sachs, 2007; Ward et al., 2018). Additionally, treatment of mdx mice with the stretch-activated channel blocker, Streptomycin, improved muscle force and reduced membrane permeability (Whitehead et al., 2006). Plans for the use of MSC blockers for clinical trials are in progress.

A second mediator of Ca\textsuperscript{2+} influx in the muscle is store-operated Ca\textsuperscript{2+} entry (SOCE) machinery. Cells hold internal calcium stores which calcium is released from in response to specific stressors (Brotto et al., 2004; Thornton et al., 2011; Zhao et al., 2005). The SOCE machinery is composed of Ca\textsuperscript{2+} sensors, stromal interaction molecule 1 (STIM1) (Liou et al., 2005), that can translocate from the endoplasmic/sarcoplasmic reticulum (ER/SR) membrane to the plasma membrane in response to depletion of the intracellular Ca\textsuperscript{2+} stores (Zhang et al., 2005).

The translocation of STIM1 activates pore-forming channels such as Orai1 to permit the entrance of Ca\textsuperscript{2+} through the plasma membrane into the cytoplasm (Prakriya et al., 2006). Orai1 is upregulated in mdx mouse muscle and decreasing its activity with the BTP-2 inhibitor represses SOCE activity and Ca\textsuperscript{2+} influx. This suggests that the Orai1-SOCE pathway is a contributor to deregulated Ca\textsuperscript{2+} homeostasis in mdx muscle and that pharmacological reduction of this pathway could be beneficial in the context of DMD (Zhao et al., 2012).

Finally, the leaky Ca\textsuperscript{2+} releasing channel, ryanodine receptor (RYR), has been studied to investigate defects in Ca\textsuperscript{2+} homeostasis in the dystrophic muscles. RyR1 controls Ca\textsuperscript{2+} release from the SR into the cytoplasm of cells to induce muscle contraction. Leakiness of the RyR1 channel can cause dysfunction of muscle fibers, cellular damage and apoptosis (Bellinger et al., 2008). The RyR1 in mdx skeletal muscle is increasingly S-nitrosylated at specific cysteine residues as the dystrophic phenotype worsens, resulting in the inhibition of calstabin-1 binding to the RyR1 complex (Bellinger et al., 2009). A recent study revealed that 4 and 12 week treatment regimens

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with the RyR1 stabilizing compound, S48168/ARM210 (ARMGO Pharma), enhanced the rebinding of calstabin-1 to the RyR1 channel, improved grip strength force function and muscle fiber histology with no adverse effects (Capogrosso et al., 2018). Other calcium channel blockers such as Dilitiazem and Verapamil showed beneficial effects against muscle degeneration in mdx mice, however, these drugs have not yet reached clinical trials (Matsumura et al., 2009). Collectively, these studies demonstrate that calcium homeostasis is disrupted in DMD and that the mechanisms regulating these pathways have potential therapeutic impact for this disease.

1.6.3 Anti-Fibrotic Treatment in Dystrophic Muscles

A key player that was linked to causing muscle fibrosis in DMD models is the polypeptide transforming growth factor beta (TGF-β). The expression levels of TGF-β1, an inflammatory cytokine, is significantly elevated in mdx tissues at 6 and 9 weeks of age, particularly in the diaphragm, which directly correlated with increased levels of type 1 collagen (Gosselin et al., 2004). Inhibition of TGF-β1 with the compound decorin results in a significant reduction in the mRNA levels of diaphragm muscle type I collagen (Gosselin et al., 2004). In addition, a new study discusses that Ly6Cpos pro-inflammatory macrophages, maintain fibroblast production of collagen through the secretion of TGF-β1 in human DMD muscles. This study suggests that the activation of this pathway can be reduced by the AMPK-activating drug Metformin, which decreases TGF-β1 through the latent TGF-β-binding protein 4 (LTBP4) causing decreased fibrosis thus improving DMD muscle force. However, it is not fully understood the direct connections between TGF-β and AMPK (Gao et al., 2018). Activation of the AMPK pathway in dystrophic muscle has been shown to be beneficial for many aspects of DMD including activation of the slow oxidative myofiber program, regulation of myofiber regeneration and activation of a homologous
protein of dystrophin called utrophin (discussed in section 1.7.3) (Dial et al., 2018). As described above Metformin is currently in clinical trials to study its therapeutic potential in DMD (Hafner et al., 2016).

Other anti-fibrotic drugs in clinical trials include Halofuginone (HT-100/Akashi Therapeutics), that inhibits Smad3 phosphorylation downstream of TGF-β in mdx muscles, thus improving cardiac and skeletal muscle function (Turgeman et al., 2008). Despite the suspension of a phase II study with HT-100 due to the death of a DMD patient, it was resumed in 2017 with a modified lower dose (Escolar et al., 2016). Other TGF-β1 inhibitors being investigated in clinical trials include losartan and lisinopril (Allen et al., 2013; Lee et al., 2014). FG-3029, an anti-connective tissue growth factor (CTGF) is also being explored in a clinical trial due to its ability to block expression levels of CTGF, a factor that contributes to the accumulation of fibrotic tissue in mdx mouse skeletal muscle (Morales et al., 2013). Together, the progression of these anti-inflammatory drugs through various stages of pre-clinical and clinical trials highlights their therapeutic potential for the treatment of DMD.

1.6.4 Treating Oxidative Stress/ROS in Dystrophic Muscles

Before the discovery of the loss of dystrophin protein in DMD patients, researchers believed that DMD could result, at least in part, from oxidative stress in muscle (Bia et al., 1999; Murphy and Kehrer, 1986, 1989; Omaye and Tappel, 1974; Tidball and Wehling-Henricks, 2007). Free radicals and reactive oxygen species (ROS) are recurrently produced in healthy muscles under normal physiological conditions. High levels of free radicals can be damaging to the cell, yet, at normal levels, radicals and other oxidants play key roles in driving gene expression, modulating signaling pathways, and controlling skeletal muscle force production, as for instance, oxidative
phosphorylation to create adenosine triphosphate (ATP) (Dröge, 2002; Powers et al., 2010; Reid, 2001a, 2001b). The most common free radicals formed in cells are superoxide (O$_2^-$) and nitric oxide (NO). Superoxide is created when oxygen is transferred an incomplete number of electrons during electron transport or by a specific production from enzymes such as NADH oxidase during inflammation. Whereas NO is generated by the enzymatic action of NO synthase (NOS) (Reid, 2001b). A reduction of oxidative stress and decreased necrosis in response to antioxidant products has been strongly supported in preclinical mdx mice experiments with catalase, coenzyme Q10 (CoQ10), green tea extracts, N-acetylcysteine and resveratrol (Bjelakovic et al., 2012; Buetler et al., 2002; Call et al., 2008; Dorchies et al., 2006; Hori et al., 2011; Ljubicic et al., 2014; Selsby, 2011). In fact, CoQ10 was used in clinical trials for its antioxidant effects. In combination with corticosteroid treatment for 3 months, CoQ10 treatment resulted in an 8.5% increase of muscle strength in DMD patients (Spurney et al., 2011). However, a more recent Iranian clinical trial that treated DMD patients with CoQ10 for 6 months showed no significant improvement in performance or cardiac function (Salehi et al., 2017). Statins, LDL-cholesterol lowering drugs, also prove to strike beneficial effects by reducing oxidative stress in dystrophin-deficient muscles. An 8-month long-term treatment of Simvastatin in mdx mice highly improves oxidative stress as well as inflammation and fibrosis through NADPH oxidase 2 protein expression (Whitehead et al., 2015, 2016).

Muscle mitochondria from patients with DMD reveal impaired oxidative phosphorylation (Scholte et al., 1985) and altered neuronal NOS, which is thought to contribute to muscle damage in this disease (Brenman et al., 1995; Grozdanovic et al., 1996). A pilot trial with L-arginine and metformin exhibited an increase in NOS expression and other mitochondrial proteins resulting in beneficial effects on muscular metabolism in the skeletal muscle tissue of patients with DMD in
vitro and in vivo (Hafner et al., 2016). A phase III clinical study of Metformin and L-arginine in DMD patients is now completed with pending results (NCT01995032). This shows that combinatorial treatment of drugs that could improve different symptoms of DMD patients or have a synergistic effect could be an attractive method to treat DMD.

1.6.5 Targeting Endogenous Genes to Restructure the DAPC at the Sarcolemma

Upregulation of endogenous genes to compensate for the lack of dystrophin is a particularly attractive approach for DMD therapeutics. This method targets genes that are naturally expressed in the dystrophic muscle, thus, bypassing the issues of bad tissue uptake as well as introducing dystrophin and inducing an immune response as observed with some gene and cell therapies (Lim et al., 2018; Mah, 2016; Mendell et al., 2010; Okada and Takeda, 2013; Wang et al., 2011b).

Such endogenous genes include transmembrane proteins such as sarcospan (Gibbs et al., 2016; Marshall and Crosbie-Watson, 2013; Marshall et al., 2015), and α7-integrin (Burkin et al., 2005; Heller et al., 2013, 2015) that when upregulate, improve dystrophic muscle fiber membrane stability and integrity. Several studies demonstrate that the overexpression of these transmembrane proteins in mdx mice ameliorates dystrophic pathology and restores muscle cell binding to laminin (Burkin et al., 2005; Heller et al., 2013, 2015). Upregulation of GalNac transferase (specifically GALGT2) has been shown to stimulate glycosylation of DAPC member α-dystroglycan as well as important DAPC and synaptic binding members which includes: laminin-α4, laminin-α5, sarcoglycans, dystrobrevins and utrophin (Chicoine et al., 2014; Martin et al., 2009; Nguyen et al., 2002; Xia et al., 2002). A clinical trial aiming to perform gene delivery of GALGT2 (rAAVrh74.MCK.GALGT2) in DMD patients is presently recruiting participants
These studies demonstrate the importance of targeting members of the DAPC to repair integrity and proper function of DMD skeletal muscle.

As mentioned above, utrophin is a homologue of dystrophin. Utrophin is an interesting candidate to stimulate in dystrophic muscles since it can compensate for the absence of dystrophin protein. The upregulation of the utrophin protein has been shown, to have beneficial effects on the performance, fiber integrity and morphology of dystrophic muscles. Many laboratories including our own have focused their efforts in increasing utrophin through gene expression or pharmacological stimulation as a therapeutic approach for the treatment of DMD (Krag et al., 2004; Ljubicic and Jasmin, 2015; Ljubicic et al., 2011, 2014; Miura et al., 2009; Moorwood et al., 2011; Tinsley et al., 1998, 1996; Voisin et al., 2005). This will be thoroughly discussed below.

1.7 Utrophin Upregulation as a Therapy for DMD

Utrophin is a large cytoskeletal protein of 395 kDa encoded by a 13kb transcript which consists of 74 exons and is located on chromosome 6q24 (Love et al., 1989; Pozzoli et al., 2002). It has two distinct promoters (A and B) that produce two utrophin mRNAs that vary at their 5’ends thus resulting in two identical functional proteins with 31 amino acids difference at the N-terminal (Baby et al., 2010; Burton et al., 1999; Dennis et al., 1996a; Pearce et al., 1993) (Figure 2). Five novel 5’utrophin splice variants were also recently identified in human adult and embryonic tissues (A’, B’, C, D and F), however these have yet to be characterized (Perkins and Davies, 2018). The two most studied utrophin isoforms, A and B, differ in their tissue localization. Utrophin A is expressed primarily at the myotendinous and neuromuscular junction (NMJ), in neurons, astrocytes as well as in the brain (choroid plexus and pia mater), whereas utrophin B is primarily localized in vasculature (Baby et al., 2010; Burton et al., 1999; Dennis et al., 1996; Nguyen et al.,
1991; Weir et al., 2002). According to the human protein Atlas and others, utrophin is also expressed in the kidneys, retinal glial cells, lungs (Haenggi and Fritschy, 2006; Matsumura et al., 1993; Raats et al., 2000) as well as highly expressed in the thyroid gland and the prostate (The Human Protein Atlas: https://www.proteinatlas.org). However, details about which isoform is expressed is yet to be described in these tissues. Utrophin A displays a varying localization pattern in muscle through embryonic to adult development. In fact, utrophin is expressed at the sarcolemma during embryogenesis, reaching maximal expression levels at approximately 17 weeks’ gestation and gradually decreasing to negligible levels at week 26, which coincides with increasing levels of dystrophin (Clerk et al., 1993; Lin and Burgunder, 2000). In adult tissues, utrophin A is mostly restricted to the NMJ but in regenerating muscles, utrophin A is expressed at the sarcolemma as a part of the repair process (Helliwell et al., 1992; Karpati et al., 1993; Khurana et al., 1991). More interestingly, unlike isoform B, the utrophin A isoform is up-regulated in dystrophin-deficient muscle fibers and in part, localized at the sarcolemma (Weir et al., 2002). Thus, these studies suggest that utrophin A may play a role similar to that of dystrophin during certain developmental stages and could compensate for the loss of dystrophin in DMD.
Figure 2. Utrophin A and B isoforms

A) The two isoforms of utrophin (A and B) have distinct promoters and 5′UTR regions. Utrophin A transcript is driven by a CpG island-containing promoter and the utrophin B transcript is driven by a TATA motif-containing promoter. The utrophin B promoter is located within the second intron of the utrophin gene resulting in a unique 5′ end. B) The utrophin A transcript contains an IRES between nucleotides 71 and 152 in its 5′UTR (560 nucleotides long). Additionally, there are AU-rich elements (ARE) in the area within nucleotides 332 and 596 of the 3′UTR of utrophin A mRNA responsible for controlling stability of utrophin A. C) Translation of utrophin A and B transcripts result in similar size proteins of 395 kDa, comprising of the same domains but differ at their N-terminal area.
1.7.1 Utrophin A Functions as a Surrogate for Dystrophin

Sequence similarities between utrophin and dystrophin indicates the potential of utrophin to compensate for dystrophin (Pearce et al., 1993; Pozzoli et al., 2002). Utrophin has similar protein binding functions to dystrophin and has approximately 80% sequence homology in the actin binding domain and the carboxy terminus including the cysteine-rich domain (Blake et al., 1992; Love et al., 1989; Pearce et al., 1993; Tinsley et al., 1992). In addition, utrophin has been shown to associate to F-actin filaments with its N-terminal actin-binding domain and interacts with members of DAPC, notably α-syntrophin, β-dystroglycan and α-dystrobrevin-1 at its C-terminal area (Ishikawa-Sakurai et al., 2004; Kramarcy and Sealock, 2000; Perkins and Davies, 2002; Peters et al., 1998; Winder et al., 1995) (Figure 3). The major structural difference between dystrophin and utrophin is in the rod domain at an identity less than 30%, where utrophin is missing repeats 15 and 19 as well as two hinge regions (Pearce et al., 1993; Winder et al., 1995a). These differences could explain why utrophin is unable to compensate for all of dystrophin’s functions. For instance, transgenic utrophin overexpression in mdx mouse models does not restore the localization of nNOS to the sarcolemma of muscle fibers, whereas dystrophin can bind nNOS to promote angiogenesis (Lai et al., 2009; Li et al., 2010). Additionally, unlike dystrophin, utrophin is unable to bind microtubules to form an organized lattice at the sarcolemma which also protects against contraction-induced injury (Belanto et al., 2014; Prins et al., 2009; Randazzo et al., 2013) (Figure 3). It is also unknown if utrophin can compensate for dystrophin in dystrophic satellite cells where it regulates their polarity and asymmetric division (Dumont et al., 2015). However, utrophin has been shown to interact with the polarity-regulating kinase partitioning-defective 1b (PAR-1b) (also known as MARK2), thus, utrophin may in fact be able to regulate cell polarity (Yamashita et al., 2010).
Despite these differences, increasing utrophin A (and not B) expression levels at the sarcolemma of dystrophic muscles has proven to be an efficient approach to compensate for the lack of dystrophin (Tinsley et al., 1998). Due to the lack of key cis-acting sequences in utrophin B promoter that are accountable for the mediation of transcriptional enhancement of utrophin A at the sarcolemma and perhaps its altered actin binding domain at the N-terminal area does not make utrophin B a good candidate to replace dystrophin in dystrophic muscles (Burton et al., 1999; Weir et al., 2002). In dystrophic muscles, there is a slight increase in utrophin A at the sarcolemma, that occurs naturally as part of the repair process (Arechavala-Gomeza et al., 2010; Helliwell et al., 1992; Schofield et al., 1995). Transgenic mice overexpressing a truncated utrophin transgene using the human skeletal α-actin (HSA) promoter improved the dystrophic phenotype of mdx mice significantly, which includes a decrease of CK levels, a reduction of centrally nucleated myofibers (indicative of fewer regenerating fibers), as well as a rescue of utrophin A and DAPC members at the sarcolemma (Tinsley et al., 1996). Transgenic mouse models expressing full-length utrophin elicited full recovery of mechanical functions of the diaphragm with only a 2-Fold induction of utrophin as compared to the mdx control and with no adverse effects on non-muscle cells (Fisher et al., 2001; Tinsley et al., 1998). As little as a 1.5-fold increase of utrophin can be beneficial as long as the localization of utrophin is at the level of the sarcolemma in dystrophic muscle fibers (Tinsley et al., 1998). Furthermore, a recent study shows that AAV9-mediated micro-utrophin expression can also strongly alleviate the cardiac defects of a more severe mdx mouse model (Kennedy et al., 2018). Similar beneficial effects were also observed in the GRMD with AAV-mediated mini-utrophin gene transfer (Cerletti et al., 2003). Thus, it appears that utrophin A is a suitable and safe replacement for dystrophin and these studies highlight its potential as a target for DMD therapy.
Figure 3. Utrophin association with members of the DAPC at the muscle fiber sarcolemma.

Like dystrophin, utrophin A can bind DAPC members at the sarcolemma of muscle fibers to provide a link between the ECM and the intracellular cytoskeleton. Utrophin has a rod domain and four hinge regions (H1, H2, H3, and H4) and a cysteine-rich region (CR) that binds β-dystroglycan. Only the N-terminal domain (N) of utrophin A can interact with F-actin filaments (Amann et al., 1999). The main difference between utrophin and dystrophin is the lack of the spectrin-like repeats 15 and 19, and 2 hinge regions. Utrophin A associates with α-dystrobrevin, syntrophins α and β at its C-terminal domain (C). The presents of utrophin also restores binding of the sarcoglycans (α, β, δ and γ), and sarcospan at the sarcolemma as well as α-dystroglycan and laminin-2 at the ECM. The syntrophins recruit nNOS at the muscle fiber membrane with their PDZ domains to mediate blood flow to the muscle however utrophin cannot recruit nNOS at the sarcolemma via direct binding similarly to dystrophin.
1.7.2 Transcriptional Activation of Utrophin A

Key pathways that activate endogenous utrophin expression are being studied closely in order to design small molecules or identify drugs that could stimulate these pathways to upregulate utrophin in dystrophic muscles. Thus, researchers have looked into the regulatory mechanisms mediating utrophin expression in skeletal muscle and found that utrophin A has two key promoter regions containing an E-box and N-box motif that drive utrophin A transcription during myogenic differentiation and synapse-specific expression at neuromuscular junction (Dennis et al., 1996; Gramolini and Jasmin, 1999; Gramolini et al., 1997, 1999a; Khurana et al., 1999). The N-box motif, shown to be important in post-synaptic expression of utrophin A, is bound by ets-related transcription factors GA-binding protein α (GABPα) and β, to induce utrophin A transcription (Dennis et al., 1996; Gramolini et al., 1997). The presence of several binding motifs for ets-transcription factors are also conserved in the utrophin B promoter (Briguet et al., 2003). The activation of both GABPα and β is mediated by an extracellular signal-related kinase (ERK) pathway via the nerve-derived growth factor heregulin (Gramolini et al., 1999a; Khurana et al., 1999). The utrophin A promoter also contains sites that are targeted by Sp1 and Sp3 zinc finger-containing transcription factors, which together with GABP, activate transcription (Dennis et al., 1996; Galvagni et al., 2001; Gyrd-Hansen et al., 2002). In parallel, another pathway driving the synaptic utrophin A expression consists of calcineurin (CN)/Nuclear factor of activated T-cells (NFAT) (which can bind to specific NFAT sites at the utrophin A promoter), cooperatively with GABP and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (Angus et al., 2005; Chakkalakal et al., 2003; Dennis et al., 1996). Moreover, transcriptional repression of E2F transcription factor 1 (E2F1) results in de-repression of PGC-1α resulting in an increase of utrophin A (Blanchet et al., 2012). The E-box motif in the utrophin A promoter is a binding site for...
myogenic regulatory factors such as basic helix-loop-helix proteins, myogenin and MyoD which enhances utrophin A expression during myogenic differentiation (Gramolini and Jasmin, 1999; Perkins et al., 2001). In addition, muscle regeneration activates the utrophin A promoter via an AP-1 sensitive intronic enhancer site (Galvagni et al., 2002; Stocksley et al., 2005). These pathways are described in detail in figure 4. Thus, activation of these signaling pathways targeting utrophin A promoter, is an attractive approach to upregulate utrophin A expression in DMD muscles.

1.7.3 Targeting a Slow Muscle-Fiber Type Switch in Dystrophic Muscle

Gene and protein expression highly differ between a fast glycolytic and a slow oxidative muscle fiber type (Galpin et al., 2012; Kang et al., 2005). For instance, there is an increase in structural proteins including dystrophin (Chopard et al., 2000; Ho-Kim and Rogers, 1992) and spectrin (Menold and Repasky, 1984; Williams et al., 2000) in slow muscle fibers. Muscle fiber type composition strongly influences muscle performance, human locomotion and athletic performance as well as different disorders such as sarcopenia, muscle wasting, and muscle-associated metabolic disorders (Galpin et al., 2012). In DMD, slow muscle fibers present with reduced damage compared to fast muscles (Webster et al., 1988). This might be in part a result of increased endogenous utrophin A expression in slow muscle fibers, thus having protective effect on the integrity of the muscle fibers during contraction (Consolino and Brooks, 2004; Gramolini et al., 2001a; Moens et al., 1993). In fact, a variety of signaling molecules involved in stimulating the slow oxidative myogenic program in DMD mouse models have been shown to influence utrophin A expression in parallel through transcriptional events (Figure 4). This includes CN/NFAT (Chakkalakal et al., 2003; Chin et al., 1998; Olson and Williams, 2000), AMP-activated
protein kinase (AMPK) (Al-Rewashdy et al., 2015; Bergeron et al., 2001; Fillmore et al., 2010; Jørgensen et al., 2007; Lira et al., 2010; Ljubicic et al., 2011, 2012), as well as co-transcription factors PGC-1α (Akimoto et al., 2005; Al-Rewashdy et al., 2015; Lin et al., 2002a; Lira et al., 2010a; Ljubicic et al., 2011, 2012), peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) (Luquet et al., 2003; Miura et al., 2009; Wang et al., 2004) and sirtuin 1 (SIRT1) (Gordon et al., 2013; Ljubicic et al., 2014; Vinciguerra et al., 2010). Indeed, upregulated extrasynaptic expression of utrophin A in slow muscles is under the control of the calcineurin/NFAT signaling pathway (Chakkalakal et al., 2003). When crossing a transgenic mouse expressing high calcineurin activity with an mdx mouse, a fiber type switch to a slow phenotype in fast twitch muscles occur, accompanied by an amelioration of sarcolemmal integrity and overall dystrophic phenotype (Chakkalakal et al., 2004; Stupka et al., 2004).

The AMPK pathway has also been intensely studied over the years for its ability to phosphorylate downstream targets to drive cellular energy homeostasis as well as to maintain and remodel the skeletal muscle (Dial et al., 2018; Kjøbsted et al., 2018; Ljubicic and Jasmin, 2013; Mounier et al., 2015). Stimulating AMPK expression, either with a constitutively active AMPK mutant or with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), demonstrates a shift toward slower myosin heavy-chain composition (a marker of slow muscle fibers) and increased mitochondrial biogenesis in skeletal muscle (Garcia-Roves et al., 2008; Jørgensen et al., 2007). In addition a loss of folliculin interacting protein-1 (Fnip1) results in AMPK activation of PGC-1α (Suwa et al., 2003) and transcriptional activation of utrophin A in a variety of animals including DMD mouse models (Al-Rewashdy et al., 2015; Ljubicic et al., 2011, 2012; Reyes et al., 2015). Collectively these studies show that promoting the slow oxidative machinery may prove to be an efficient way to increase utrophin A expression levels.
Figure 4. Key signaling pathways involved in utrophin A transcription. Pharmacological activation of AMPK and Sirt-1 results in deacetylation (D) or phosphorylation (P) of co-transcription factor PGC-1α in collaboration with either PPARβ/δ or GABPα/β to activate utrophin A promoter at the PPRE or N-box sites. Direct stimulation of PPARβ/δ also results in utrophin A transcriptional activation. Interaction of heregulin to its receptor (HER) induces phosphorylation of ERK, resulting in stimulation of GABPα/β, leading to N-box binding as well as activation of the utrophin promoter with Sp1 and Sp3. Binding of heregulin also results in ets-2 repressor factor (ERF) de-repression of the N-box. Ca2+ and calmodulin-dependent calcineurin activates NFAT which can interact with an NFAT binding site. The myogenic regulatory factors (MRF)s can enhance the utrophin A expression, via the E-box, during myogenic differentiation and the transcription factor AP-1, via the AP-1 intronic site, throughout muscle fiber regeneration. Engineered synthetic zinc finger proteins such as Jazz are designed to target the utrophin promoter directly at the sequence 5’-GCT GCT GCG-3’. Other activators of utrophin A transcription include Ezutromid, Okadaic Acid, Nabumetone and L-arginine.
1.7.4 Post-Transcriptional Activation of Utrophin A

Post-transcriptional regulation plays an important role in modulating expression and localization of a target mRNA in tissues (Blencowe et al., 2009). When investigating the ~2 kb 3’untranslated region (UTR) of utrophin A, researchers have identified key cis elements and binding sites that are likely involved in post-transcriptional regulation of utrophin A. These post-transcriptional events can be separated in three main themes: 1) direct repression of utrophin A by binding utrophin A transcripts at the 3’UTR, 2) stabilization of utrophin A transcripts by inhibiting ARE-mediated decay and 3) Directing localization of utrophin A at the sarcolemma resulting in utrophin A protein stabilization. These mechanisms are further described at figure 5.

1.7.4.1 Post-Transcriptional Repression of Utrophin A Transcripts

Recent studies have revealed that the 3’UTR of utrophin A contains predicted microRNA (miRNA) binding sites whom are responsible for the repression of utrophin A. In fact, using miRNA binding site-blocking oligomers can de-repress this translational inhibition. In these studies, six miRNAs (miR-206, let-7c, miR-150, miR-196b, miR-296-5p, miR-133b) were identified to mediate the repression of utrophin A either by inhibiting utrophin A translation process or by inducing mRNA degradation (Basu et al., 2011; Mishra et al., 2017; Rosenberg et al., 2006). Thus, therapies aimed at using small molecules to compete with specific miRNA binding sites could be a potential method to increase utrophin A expression (Mishra et al., 2017).

1.7.4.2 Targeting Utrophin A mRNA Stability

Post-transcriptional regulation influences expression of several mRNAs in skeletal muscle (Chakkalakal and Jasmin, 2003). mRNAs encoding myogenic mediators MyoD and myogenin as
well as acetylcholinesterase (AChE) and α-dystrobrevin 1 have been shown to be regulated at the post-transcriptional level through mRNA stability (Apponi et al., 2011). Interestingly, our lab revealed that greater mRNA stability in slow oxidative muscles compared to fast muscles may account for the increased expression levels of utrophin A, suggesting the involvement of post-transcriptional events in the regulation of utrophin A expression (Gramolini et al., 2001a).

The 3’UTR of utrophin has a series of cis elements, which include conserved AU-rich elements (ARE) (Apponi et al., 2011; Bakheet et al., 2003; Chakkalakal et al., 2008; Gramolini et al., 2001b). Our laboratory showed that the ARE region at the 3’UTR was responsible for the lower levels of utrophin by suppressing the expression of reporter mRNAs (Chakkalakal et al., 2008). AREs are the most common cis-element and are responsible for rapid mRNA decay in mammalian cells. ARE-binding proteins (AUBP) such as KH-type splicing regulatory protein (KSRP), Tristetraprolin (TTP) (Brooks and Blackshear, 2013), human antigen-related protein (HuR) (Silanes et al., 2004) and AUF1 (DeMaria and Brewer, 1996) can bind the ARE elements and promote either decay or stabilization of a variety of mRNAs. Amirouche et al., show that KSRP binds preferentially to the 3’UTR AREs of utrophin A mRNA and promotes its decay in cultured myogenic cells as well as in muscles from wild-type and mdx mice (Amirouche et al., 2013). KSRP comprises four KH homology (KH) motifs that interact with AREs, the exosome and the deadenylase poly(A) specific ribonuclease (PARN) to promote rapid decay of its target ARE-containing mRNAs (Apponi et al., 2011; Gherzi et al., 2004). Exonucleolytic cleavage of mRNAs is driven by the exosome which contains a large multi-protein complex of different exonucleases (Eberhardt et al., 2007). The activity of AUBPs, including KSRP, is usually modulated by post-translational modifications such as phosphorylation. One such kinase is p38 MAPK, this protein can phosphorylate KSRP which compromises its binding and attenuates its mRNA destabilizing
function (Briata et al., 2005). Our laboratory and others has shown that in fact, phosphorylation by p38 MAPK promotes sequestration of KSRP by 14-3-3 (Díaz-Moreno et al., 2009) and reduces its functional availability to bind utrophin’s 3’UTR AREs, thus promoting its stabilization (Amirouche et al., 2013). Furthermore, the anticoagulant FDA-approved drug Heparin can activate p38 MAPK and increase utrophin A in the diaphragm muscle of mdx mice following a short 10 day treatment (Amirouche et al., 2013). In parallel, our lab showed that miR-206 can also inhibit kinases such as the KSRP that induce utrophin A mRNA decay (Amirouche et al., 2014). Thus, miR-206 can diverge between direct repression of utrophin A and activation of utrophin A through the inhibition of its destabilizers.

Collectively, this suggests that pharmacological approaches targeting the p38 MAPK/ KSRP pathway could be a promising method to increase utrophin A expression levels in dystrophic muscles of DMD patients.

1.7.4.3 Targeting Utrophin A Protein Stability

Stabilizing utrophin A at the sarcolemma is also an alternative way to insure proper localized expression of utrophin. One such protein involved in stabilizing utrophin A is biglycan. The proteoglycan biglycan is important for the maintenance of muscle cell integrity and is highly expressed at the extracellular matrix of regenerating muscle fibers (Casar et al., 2004) as well as in DMD and mdx skeletal muscles (Bowe et al., 2000; Zanotti et al., 2005). This protein contributes to the transcription and structural management of the DAPC such as nNOS (Mercado et al., 2006), and recruits utrophin to the sarcolemma (Amenta et al., 2011). A single systemic dose of recombinant human biglycan in mdx mice resulted in ex vivo functional improvements and a 2.5-fold increase in utrophin protein expression without any changes in mRNA levels, suggesting post-
transcriptional or translational events (Amenta et al., 2011). Even though the mechanism in which biglycan increases utrophin A is unknown, the human recombinant biglycan (TVN-102, Tivorsan) is in preparation for clinical trials. A three-fold increase of the protein sarcospan has also proven to increase utrophin A stability at the muscle fiber membrane by increasing transportation of utrophin and DAPC member, α-dystroglycan, from the endoplasmic reticulum and the Golgi to the sarcolemma (Gibbs et al., 2016). These results suggest that pharmacological activation of key pathways involved in post-transcriptional regulation of utrophin A prove to be a beneficial approach for the treatment of DMD.

Ras homolog gene family, member A (RhoA) GTPase, a protein involved in myogenesis induction, is another player thought to be involved in post-transcriptional regulation of utrophin A. When expressing a constitutively active form of RhoA GTPase (RhoAV14) in C2C12 muscle cells, the utrophin promoter A is transcriptionally activated, along significantly enhanced utrophin A protein stability (Bonet-Kerrache et al., 2005). The role of RhoA in post-transcriptional activation of utrophin A is misunderstood, yet, it is suggested that the most likely mechanism is protection from protein degradation (Bonet-Kerrache et al., 2005; Laufs and Liao, 1998). L-arginine which also activates the utrophin A promoter (as described above) might likewise stabilize utrophin A protein. It’s suggested that L-arginine’s stimulation of NO- could inhibit calpain’s proteolysis effects on utrophin A (Vianello et al., 2013; Waheed et al., 2005).

Thus, increased protein expression of utrophin A at the sarcolemma of muscle fibers might be in part due to stabilization of utrophin A protein through post transcriptional events.
Figure 5. Post-transcriptional regulation of utrophin A. A) miRNA binding sites at the 3’UTR of utrophin A mRNA drives the repression of utrophin A expression either by silencing or repression its translation. However, many post-transcriptional events result in utrophin A upregulation: B) p38 activation by Heparin induces a reduction in the functional availability of the RNA-binding protein KSRP, which is involved in ARE-mediated decay of utrophin A transcripts. This decrease in the functional availability of KSRP is achieved by either 1) an increase in its phosphorylation status which promotes its sequestration by the regulatory protein 14-3-3; and 2) a decrease in its expression such as repression by mir206. Thus, reducing KSRP’s induction of ARE-mediated decay (AREMD) and promoting utrophin A transcript stability. C) Utrophin A protein stability by RhoA and L-arginine might be in part through inhibition of calpain activity on utrophin A proteolysis, however this is still miss-understood. D) The stabilization of utrophin is also effectuated by biglycan which recruits utrophin-associated protein complex members α-dystroglycan and utrophin A at the sarcolemma.
1.7.5 Translational Activation of Utrophin A

Evidence of translational regulation of utrophin A arose from studies where the augmentation of utrophin A protein expression levels in muscle was not accompanied by an increase in utrophin A mRNA levels (Gramolini et al., 1999b, 2001a; Pasquini et al., 1995; Roma et al., 2004; Weir et al., 2002). The 5’ UTR of mRNAs, a region upstream from the initiation codon, serves as an entry point for ribosome binding hence mediating translation of the transcript. Utrophin A has a long 5’UTR predicted at 560 nucleotides for humans (508 for murine), that expands from the transcriptional start site, through exon 1A and 2A to the translation initiation site for utrophin A (Burton et al., 1999; Dennis et al., 1996) (Figure 2). The long utrophin A 5’UTR is also predicted to have a high degree of secondary structure and is translationally repressed through both its 5’UTR and 3’UTR in myoblast muscle cells (Basu et al., 2011; Miura et al., 2005; Zuker, 2003). As the repression by the 3’-UTR has been attributed to miRNAs and ARE-mediated degradation (described above), the exact inhibitory mechanism operated through 5’-UTR is misunderstood (Basu et al., 2011).

1.7.5.1 Cap-Dependent Translation of Utrophin A

Recent studies have shown that utrophin A can be translated through both cap-dependent and IRES-dependent translation (Ghosh and Basu, 2015; Miura et al., 2005, 2008, 2010). The rate-limiting step of cap-dependent translation initiation is the binding of a eukaryotic initiation factor (EIF) 4F protein complex to a 7-methylguanylate cap (m7G), also known as the 5’cap, of all nuclear-encoded eukaryotic mRNAs including utrophin A. Before this occurs, a series of EIFs form a pre-initiation complex. Each EIF plays a critical role such as eIF3 which separates 40S and 60S ribosomal subunits, eIF2-GTP that delivers the first transfer RNA (tRNA-Met) to the
ribosome and EIF4F complex, which recruits the pre-initiation complex to utrophin’s mRNA. The poly(A) binding protein (PABP) associates with EIF4F and binds to the poly(A) tail of the mRNA to promote circularization of the mRNA to facilitate translation. EIF4F is composed of three subunits: eIF4A (an ATP-dependent RNA helicase), eIF4E (binds the \( \text{m}^7\text{G} \) cap) and eIF4G (holds the complex together). The pre-initiation complex starts by scanning the utrophin transcript towards the 3’ end until it detects the first AUG where it will bind and hydrolysis of eIF2-GTP will arise. This process locks the complex into place allowing for elongation factors to bring in the next tRNAs, permitting peptidyl transferase ribozymes to catalyze peptide synthesis, thus generating a specific protein such as utrophin A protein (Baird et al., 2006; Holcik et al., 2000; James and Smyth, 2018) (Figure 6A).

Cap-dependent translation can be repressed in a variety of ways including: cleavage of eIF4G by viruses, thus, preventing eIF4E to bind the \( \text{m}^7\text{G} \) cap; phosphorylation of eIF2, preventing recruitment of tRNAs to the ribosome; and increasing the eIF4E-binding protein (4E-BP) which inhibits eIF4E’s ability to bind eIF4G (Bogorad et al., 2017; Gradi et al., 1998; Holcik et al., 2000; Müller et al., 2013; Svitkin et al., 1999). In fact, many physiological, pathophysiological and stress conditions including cell cycle, cardiac hypertrophy, hypoxia as well as apoptosis, lead to inhibition of cap-dependent translation and cause a substantial increase in cellular internal ribosome entry site (IRES)-mediated translation (Graber and Holcik, 2007; Komar and Hatzoglou, 2005, 2011; Wada et al., 1996) (Figure 5B).

### 1.7.5.2 IRES-Dependent Translation

A rising number of mRNAs are believed to possess IRESs which permits translation of a transcript independently of its 5’end cap. It is suggested that conventional scanning from the 5’-
end is not efficient for most IRES-containing cellular mRNAs because their 5′UTRs are typically long, GC-rich and highly structured as seen with utrophin’s 5′UTR (Ghosh and Basu, 2015; Komar and Hatzoglou, 2011; Miura et al., 2005). In cap-independent translation, the ribosome binds the IRES site directly and allows continued or promoted expression of a protein when cap-dependent translation is suppressed (Komar and Hatzoglou, 2005, 2011). IRES elements are believed to participate in various interactions with components of the translational machinery including the canonical initiation factors, IRES trans-acting factors (ITAF)s (described below) and the 40S ribosomal subunit (Komar and Hatzoglou, 2011) (Figure 6B). Previous reports also demonstrate that the proteins controlling IRES-dependent translation initiation are modulated by their subcellular localization (Lewis and Holcik, 2008). However, the regulation of protein translation by IRES are still largely misunderstood and requires additional studies.

With the use of a bicistronic reporter construct containing utrophin’s 5′UTR, our lab has demonstrated that in intact muscles, no utrophin A IRES activity was detected. However, a 9-fold increase of reporter activity was observed in muscles subjected to degeneration and regeneration cycles by cardiotoxin injections, thus revealing possible translational regulation of utrophin regenerating muscles (Miura et al., 2005). In fact, this finding was recently confirmed by others (Ghosh and Basu, 2015). The 5′UTR of utrophin contains an IRES that can stimulate utrophin A expression in muscle during muscle regeneration and glucocorticoid therapies (Miura et al., 2005, 2008). This IRES can drive translation of utrophin A strictly in skeletal muscles (Miura et al., 2010). Recent findings revealed an IRES site within exon 5 of the dystrophin gene that generates a functional N-terminally truncated dystrophin protein, which is also glucocorticoid inducible (Wein et al., 2014). However, IRESs are not defined by a consensus sequence or RNA structure
(Baird et al., 2006), thus, despite the many similarities between utrophin A and dystrophin the regulation of dystrophin’s IRES-dependent translation may not be comparable to utrophin’s.
Figure 6. Cap-dependent and cap-independent translation initiation in eukaryotic cells. A) Cap-dependent translation requires the machinery of canonical initiation factors. eIF3 keeps 40S and 60S ribosomal subunits separated by associating to 40S, eIF2-GTP recruits the first tRNA (tRNA-Met) to the P site of the ribosome. eIF4F complex (eIF4E, eIF4A, eIF4G) recruits the pre-initiation machinery to bind the mRNA. The pre-initiation complex binding is non-specific and scans the transcript to find the first AUG. When AUG is detected, there is hydrolysis of eIF2-GTP which locks the complex into place. EIF3 leaves allowing the 60S to bind. B) Cap independent translation is caused by many stresses including cell cycle, hypoxia, apoptosis and viral infection. Cap-dependent translation inhibition can occur when eIF4G is cleaved which prevents eIF4E to bind the cap m7G, eIF2 is phosphorylated and can’t recruit tRNAs as well as 4EBP binding to EIF4E which inhibits association to eIF4G. This suppression leads to cap-independent translation which occurs with a few canonical initiation factors (no eIFs except eIF2, or no eIF2 but all other eIFs, or no EIF4E) ITAFs and the 40S ribosomal subunit that binds the IRES site directly and allows continued or promoted expression of the protein.
1.7.5.3 IRES Trans-Acting Factors Involved in IRES-Mediated Translation

In many cases, IRES-dependent translation necessitates ITAFs, to recruit the ribosome and initiate polypeptide synthesis (Spriggs et al., 2005). ITAFs are suggested to be able to increase affinity of binding between IRESs and canonical initiation factors as well as the ribosome. In fact, ITAFs have been hypothesized to act as RNA chaperones to help the IRES primary sequence attain the appropriate conformational state to promote ribosome binding (Pickering et al., 2004). Interestingly, it has been reported that some IRESs require a combination of two or three ITAFs to reach efficient translational activity (Cobbold et al., 2008; Hunt and Jackson, 1999; Hunt et al., 1999; Pilipenko et al., 2000). Examples of some of the most studied ITAFs include: human La autoantigen (LA), Upstream of N-ras (Unr), The poly(rC) binding protein-2 (PCBP2) and polypyrimidine tract binding protein (PTB) (Bushell et al., 2006; Cornelis et al., 2005; Costa-Mattioli et al., 2004; Damiano et al., 2013; Dave et al., 2017; Hunt and Jackson, 1999; Hunt et al., 1999; Jo et al., 2008; Kim et al., 2018; Pilipenko et al., 2000; Shi et al., 2016; Tinton et al., 2005). However, many more ITAFs have been identified as described in table 1.

Our laboratory identified, with RNA-affinity chromatography experiments, the ITAF elongation factor 1A2 (eEF1A2), that binds the 5’UTR of utrophin and mediates utrophin A’s IRES-dependent translation (Miura et al., 2010). This data is in agreement with a recent study reporting that another elongation factor, eEF2, acts as an ITAF to regulate IRES-mediated translation of XIAP and FGF2 mRNAs (Argüelles et al., 2014).

There are two eEF1A isoforms: eEF1A1 and eEF1A2 which share 92% sequence identity (Kahns et al., 1998). Both eEF1A isoform’s main function is regulating transport of aminoacyl-tRNA throughout translation elongation, however, eEF1A2 also plays a role in muscle where it
Table 1. Examples of IRES trans-acting factors and their target IRESs.

<table>
<thead>
<tr>
<th>ITAFs</th>
<th>Target IRESs</th>
<th>References</th>
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<tbody>
<tr>
<td>Argonaute 2</td>
<td>Enterovirus 71</td>
<td>(Lin et al., 2015)</td>
</tr>
<tr>
<td>eEF1A2</td>
<td>Utrophin A</td>
<td>(Miura et al., 2010)</td>
</tr>
<tr>
<td>eEF2</td>
<td>XIAP, FGF2</td>
<td>(Argüelles et al., 2014)</td>
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<tr>
<td>eIF-2</td>
<td>PITSLRE kinase</td>
<td>(Tinton et al., 2005)</td>
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<tr>
<td>hnRNPA1</td>
<td>Cyclin D1, c-myc, sterol-regulatory-element-binding protein 1 (SREBP-1)</td>
<td>(Damiano et al., 2013; Jo et al., 2008; Shi et al., 2016)</td>
</tr>
<tr>
<td>Human antigen R (HuR)</td>
<td>Enterovirus 71, Type I insulin-like growth factor receptor (IGF-IR)</td>
<td>(Lin et al., 2015; Meng et al., 2005)</td>
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<tr>
<td>ITAF(45)</td>
<td>Theiler's murine encephalomyelitis virus (TMEV)</td>
<td>(Pilipenko et al., 2000)</td>
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<tr>
<td>La autoantigen</td>
<td>Hepatitis C virus (HCV), poliovirus, XIAP, BIP</td>
<td>(Costa-Mattioli et al., 2004; Holcik and Korneluk, 2000; Kim et al., 2001)</td>
</tr>
<tr>
<td>Poly(rC) binding protein-2 (PCBP2)</td>
<td>HCV, Hairless (HR), picornavirus type I</td>
<td>(Fujimura et al., 2008; Kim et al., 2018; Walter et al., 1999; Wang et al., 2011a)</td>
</tr>
<tr>
<td>Polypyrimidine tract binding protein (PTB)</td>
<td>TMEV, UNR, Apoptosis-related mRNAs, Coxsackievirus B3 (CVB3)</td>
<td>(Bushell et al., 2006; Cornelis et al., 2005; Dave et al., 2017; Pilipenko et al., 2000)</td>
</tr>
<tr>
<td>Polypyrimidine tract binding protein-associated splicing factor (PSF), G-rich RNA sequence binding factor 1 (GRSF), Y-box binding protein 1 (YB1)</td>
<td>c-myc</td>
<td>(Cobbold et al., 2008)</td>
</tr>
<tr>
<td>Upstream of N-ras (UNR)</td>
<td>Human rhinovirus, PITSLRE kinase</td>
<td>(Hunt et al., 1999; Tinton et al., 2005)</td>
</tr>
<tr>
<td>Vasohibin1</td>
<td>Fibroblast Growth Factor 1 (FGF1)</td>
<td>(Prats et al., 2018)</td>
</tr>
</tbody>
</table>
prevents apoptosis (Ruest et al., 2002). Several other functions have also been attributed to eEF1A including, interaction with the cytoskeleton, binding and bundling of actin filaments as well as inducing a severing effect on microtubules (Lopez-Valenzuela et al., 2003; Mateyak and Kinzy, 2010). In contrast with eEF1A1 that is generally expressed ubiquitously, eEF1A2 is specifically expressed in skeletal muscle, heart and brain (Knudsen et al., 1993a; Lee et al., 1992). This suggests that eEF1a2 could play a distinct role in muscles and that identifying pharmacological compounds that would target eEF1A2 to increase endogenous levels of utrophin A expression could serve as a drug-based therapy to treat DMD.

1.8 Pharmacological Stimulation of Utrophin A for the Treatment of DMD

Pharmacological treatment has many benefits over other therapies including little to no toxicological concerns or undesired immune responses as well as practical systemic administration and ease of delivery to the muscles (Guiraud and Davies, 2017). Increasing evidence suggests that pharmacological upregulation of utrophin A protein in dystrophic muscles is a promising and safe therapeutic strategy for the treatment of DMD. Over the years, small molecules and drugs have been developed to target utrophin A transcriptional and post-transcriptional signaling pathways (described above) in order to create a therapeutic approach to increase endogenous utrophin A expression levels in DMD dystrophic muscles.

For instance, small-molecules targeting the utrophin A promoter have been perfected to stimulate utrophin A transcription. In fact, studies are aimed at generating transgenic mice that over-express artificial zinc finger, Jazz and UtroUp proteins, which are able to specifically increase the endogenous utrophin gene at the muscular level (Corbi et al., 2000; Desantis et al., 2009; Di Certo et al., 2010; Mattei et al., 2007; Onori et al., 2013). Furthermore, a novel approach using the
CRISPR/Cas9 system to activate the utrophin A promoter is also in development. A recent study from Wojtal et al. used the Cas9 technology fused to VP16 trans-activating elements targeted to the utrophin A and B promoters, which resulted in an augmentation of utrophin in DMD myoblasts (Wojtal et al., 2016). In addition, the peptide heregulin was also shown to upregulate utrophin A’s transcription and improve the dystrophic phenotype of mdx mice. (Gramolini et al., 1999a; Krag et al., 2004). Other molecules reported to modulate the utrophin A promoter include: agrin (Gramolini et al., 1998) and L-arginine (a precursor required for nNOS-induced NO synthesis) (Chaubourt et al., 1999; Vianello et al., 2013; Voisin et al., 2005) (Figure 4).

Many drugs have also been shown to activate utrophin A promoter including Okadaic acid (Rodova et al., 2004), Nabumetone (Moorwood et al., 2011) and Ezutromid (SMT C1100) (Guiraud and Davies, 2017; Khurana and Davies, 2003; Moorwood et al., 2011, 2013; Tinsley et al., 2015, 2011). In fact, treatment with Ezutromid revealed promising preclinical results in dystrophic mice including increased membrane stability, decreased regeneration of fibers, reduced inflammation and improved overall muscle function. This drug has now advanced to phase II clinical trials (NCT02858362).

A variety of utrophin A transcriptional-activating drugs, which in parallel promote the slow oxidative program, were shown to have beneficial effects in dystrophic muscles. AMPK activating drug AICAR induces mitochondrial biogenesis in skeletal muscle (Jørgensen et al., 2007) along with the activation of PGC-1α (Suwa et al., 2003) and transcription of utrophin A in a variety of animals including DMD mouse models (Al-Rewashdy et al., 2015; Ljubicic et al., 2011, 2012; Reyes et al., 2015). Furthermore, a fiber-type switch and beneficial effect on dystrophic muscles was also observed following 3-6 week treatments of DMD mouse models with drugs such as GW501516, metformin and resveratrol along with transcriptional stimulation of utrophin A by
activating the key regulatory factors: PPARβ/δ, AMPK, SIRT1 and PGC-1α as summarized in figure 4 (Al-Rewashdy et al., 2015; Gordon et al., 2013; Langone et al., 2014; Ljubicic and Jasmin, 2015; Ljubicic et al., 2011, 2014; Miura et al., 2009) (Figure 4).

Although many pharmacological approaches aimed at stimulating transcription of utrophin A have been established, only a few drugs and small molecules have been shown to upregulate utrophin A through post-transcriptional and translational measures. For example, a specialized oligonucleotide was designed to bind the utrophin 3'UTR and compete with let-7c miRNA in order to prevent repression of utrophin A (Mishra et al., 2017). In addition, as described above, short pharmacological treatments of mdx mice with heparin, biglycan and L-arginine results in stabilization of either utrophin A transcripts or utrophin A protein resulting in increased utrophin A expression levels in dystrophic muscles (Amenta et al., 2011; Amirouche et al., 2013; Vianello et al., 2013; Waheed et al., 2005).

In summary, many pharmacological approaches are successfully being developed to stimulate utrophin A, which demonstrates the feasibility of this method for the treatment of DMD. The most promising utrophin A activating compounds are summarized in table 2.

1.9 Repurposing FDA-Approved Drugs

As described above, pharmacological stimulation of endogenous utrophin A in dystrophic muscles is a promising approach for the treatment of DMD. At this present time, one small molecule called ezutromid (SMT C1100), has completed phase Ia and Ib clinical trials, which were reported to be well-tolerated with no severe adverse events (Ricotti et al., 2016; Tinsley et al., 2015). The phase Ib trial was held to verify whether a variation in diet would permit a better absorption of SMT C1100 indicating that this drug might be hard to metabolize (Muntoni et al.,
Summit Therapeutics is currently carrying out a phase II clinical trial (NCT02858362). This shows that the development of a new drug is very time consuming and might present many obstacles throughout the approval process. In fact, this drug approval process can be long and difficult with under 20% of phase II trials being successful. This is often due to a lack of efficacy or safety issues that only become apparent with a greater number of patients or in longer trials (Arrowsmith, 2011; Arrowsmith and Miller, 2013; Carlisle et al., 2015).

In the past years, researchers have identified FDA-approved drugs to use in a DMD context which includes Diacerhein (Mâncio et al., 2017), Nabumetone (Moorwood et al., 2011), Safinamide (Vitiello et al., 2018), Tamoxifen (Wu et al., 2018), Simvastatin (Whitehead et al., 2015, 2016) as well as many different flavanols such as quercetin (Ballmann et al., 2015; Selsby et al., 2016). In addition, previous studies from our laboratory have exploited the off-target effects of various drugs in stimulation of utrophin A expression in muscle such as the PPARβ/δ agonist GW501516, the natural phenol Resveratrol as well as the anti-diabetic drugs Metformin and AICAR (Al-Rewashdy et al., 2015; Ljubicic and Jasmin, 2015; Ljubicic et al., 2011, 2014; Miura et al., 2009). This approach termed drug-repurposing results in faster development times into clinical trials, reduces health risks and lowers costs, making this an efficient therapeutic strategy for DMD (Ashburn and Thor, 2004; Corsello et al., 2017).
### Table 2. Promising utrophin A-activating drugs and small molecules for DMD treatment.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Animal model</th>
<th>Pathway Targeted</th>
<th>Urophin A upregulation</th>
<th>Beneficial physiological effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrin</td>
<td>C2C12 cells</td>
<td>utrophin A promoter</td>
<td>↑utrophin A transcript ↑utrophin A protein</td>
<td>↑fiber morphology ↑slow fiber switch ↑utrophin sarcolemmal localization ↑sarcolemmal integrity ↑muscle function</td>
<td>(Gramolini et al., 1998)</td>
</tr>
<tr>
<td>AICAR</td>
<td>mdx, dKO (dystrophin/utrophin)</td>
<td>AMPK/PGC-1α</td>
<td>↑utrophin A transcript ↑utrophin A protein</td>
<td>↑utrophin sarcolemmal localization ↓creatine kinase ↑fiber morphology ↑muscle function</td>
<td>(Al-Rewashdy et al., 2015; Jahnke et al., 2012; Ljubicic et al., 2011, 2012)</td>
</tr>
<tr>
<td>Biglycan</td>
<td>mdx</td>
<td>α-dystroglycan</td>
<td>↑utrophin A protein</td>
<td>↑utrophin sarcolemmal localization ↓creatine kinase ↑fiber morphology ↑muscle function</td>
<td>(Amenta et al., 2011; Bowe et al., 2000)</td>
</tr>
<tr>
<td>CT-GalNac</td>
<td>mdx</td>
<td>α-dystroglycan</td>
<td>↑utrophin A transcript ↑utrophin A protein</td>
<td>↑utrophin sarcolemmal localization ↑sarcolemmal integrity</td>
<td>(Durko et al., 2010)</td>
</tr>
<tr>
<td>Ezutromid</td>
<td>mdx, DMD patients</td>
<td>utrophin A promoter</td>
<td>↑utrophin A transcript ↑utrophin A protein</td>
<td>↑utrophin sarcolemmal localization ↑sarcolemmal integrity ↑muscle function ↓creatine kinase</td>
<td>(Guiraud and Davies, 2017; Guiraud et al., 2015; Tinsley et al., 2011)</td>
</tr>
<tr>
<td>GW501516</td>
<td>mdx</td>
<td>PPARβ/δ</td>
<td>↑utrophin A transcript ↑utrophin A protein</td>
<td>↑muscle mass ↑muscle function ↑sarcolemmal integrity ↑fiber morphology ↓inflammation ↑slow fiber switch</td>
<td>(Jahnke et al., 2012; Miura et al., 2009)</td>
</tr>
<tr>
<td>Heparin</td>
<td>mdx</td>
<td>p38MAPK/KSRP</td>
<td>↑utrophin A transcript ↑utrophin A protein</td>
<td></td>
<td>(Amirouche et al., 2013)</td>
</tr>
<tr>
<td>Heregulin</td>
<td>mdx</td>
<td>ERK/GABPa/β</td>
<td>↑utrophin A transcript ↑utrophin A protein</td>
<td>↓creatine kinase ↑fiber morphology ↑muscle function</td>
<td>(Gramolini et al., 1999a; Khurana et al., 1999; Krag et al., 2004)</td>
</tr>
<tr>
<td>Jazz</td>
<td>mdx</td>
<td>utrophin A promoter</td>
<td>↑utrophin A transcript ↑utrophin A protein</td>
<td>↑fiber morphology ↑muscle function ↑sarcolemmal integrity</td>
<td>(Corbi et al., 2000; Di Certo et al., 2010; Onori et al., 2013)</td>
</tr>
</tbody>
</table>

↑ (increased or improved) ↓ (decreased)
Table 2 (continued). Promising utrophin A-activating drugs and small molecules for DMD treatment.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Animal model</th>
<th>Pathway Targeted</th>
<th>Utrophin A upregulation</th>
<th>Beneficial physiological effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginine</td>
<td>mdx, dKO (dystrophin/utrophin)</td>
<td>NO</td>
<td>↑utrophin A transcript, ↑utrophin A protein</td>
<td>↑utrophin sarcolemmal localization, ↑sarcolemmal integrity, ↓creatine kinase, ↑muscle function</td>
<td>(Vianello et al., 2013; Voisin et al., 2005)</td>
</tr>
<tr>
<td>Metformin</td>
<td>mdx</td>
<td>AMPK</td>
<td>↑utrophin A protein</td>
<td>↓fibrosis, ↓inflammation</td>
<td>(Gosselin et al., 2004; Ljubicic and Jasmin, 2015)</td>
</tr>
<tr>
<td>Nabumetone</td>
<td>C2C12 cells</td>
<td>utrophin A promoter</td>
<td>↑utrophin A transcript, ↑utrophin A protein</td>
<td></td>
<td>(Moorwood et al., 2011)</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>C2C12 cells</td>
<td>Sp1</td>
<td>↑utrophin A transcript</td>
<td></td>
<td>(Rodova et al., 2004)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Mdx</td>
<td></td>
<td>↑utrophin A protein</td>
<td>↑sarcolemmal integrity, ↑cardiac pathology, ↑respiratory function</td>
<td>(Ballmann et al., 2015; Selsby et al., 2016)</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Mdx</td>
<td>AMPK/SIRT1</td>
<td>↑utrophin A transcript, ↑utrophin A protein</td>
<td>↑muscle mass, ↑muscle function, ↑sarcolemmal integrity, ↓oxidative damage, ↓inflammation, ↑slow fiber switch</td>
<td>(Gordon et al., 2013; Hori et al., 2011; Ljubicic et al., 2014)</td>
</tr>
</tbody>
</table>

↑ (increased or improved) ↓ (decreased)
1.10 Statement of Problem and Hypothesis

The summarized literature above demonstrates the need to continue to pursue our research on understanding the different molecular pathways involved in the progression of DMD, in order to characterize drugs or small molecules that could improve the deleterious symptoms of this disease. Despite success with the exon skipping drug Exondys51 (Eteplirsen), conditionally approved by the FDA (Irwin and Herink, 2017; Niks and Aartsma-Rus, 2017) and the 214 studies in clinical trial for the treatment of DMD, there is a need to develop therapies with high efficiency, ease of administration, and with wide applicability.

Increasing the endogenously expressed protein utrophin A in dystrophic muscles is a promising strategy for the treatment of DMD because it is a safe therapy applicable to all DMD patient and which affects all muscles. Even though mechanisms that drive transcriptional regulation of utrophin A are generally well established, passed research from our lab and others shows that the post-transcriptional and translational events also play an important role in the modulation of utrophin A expression. Overall, this project consists in identifying and characterizing FDA-approved agents to activate the pathways involved in utrophin A’s post-transcriptional/translational events. Thus, we hypothesize that repurposing FDA-approved drugs activating utrophin A, will be an efficient approach in rapidly bringing new therapeutic interventions for DMD. To test this hypothesis, we formulated the following objectives:

Objectives:

1. Explore the effects of a combinatorial treatment with FDA-approved drugs that target distinct signaling pathways, on utrophin A expression levels in mdx mice.

2. Investigate the effects of a p38 MAPK activating FDA-approved drug on the dystrophic phenotype of mdx mice and on utrophin A expression.

3. Identify and characterize FDA-approved drugs that target eEF1A2 as a potential therapeutic approach for DMD
Chapter 2
Combinatorial Therapeutic Activation with Heparin and AICAR Stimulates Additive Effects on Utrophin A Expression in Dystrophic Muscles


* These authors contributed equally to this work
Author’s contribution:

Conceived and designed the experiments: CP, AAh, BJJ. Performed the experiments: CP, AAh, TCP. Analyzed the data: CP, AAh. Provided expertise: LMB, VL, JMR, BJJ. Contributed reagents/materials/analysis tools: JMR. Wrote the Manuscript: CP, BJJ. Contributions by figure: Fig 7 (CP), Fig 8 (CP), Fig 9 (CP), Fig 10 (CP), Fig 11 (CP), Fig 12 (CP, JMR), Fig 13 (AAh), Fig 14 (CP), Fig 15 (CP, AAh), Fig 16 (AAh), Fig 17 (CP, AAh)
Abstract

Upregulation of utrophin A is an attractive therapeutic strategy for treating Duchenne Muscular Dystrophy (DMD). Over the years, several studies revealed that utrophin A is regulated by multiple transcriptional and post-transcriptional mechanisms, and that pharmacological modulation of these pathways stimulates utrophin A expression in dystrophic muscle. In particular, we recently showed that activation of p38 signaling causes an increase in the levels of utrophin A mRNAs and protein by decreasing the functional availability of the destabilizing RNA-binding protein KSRP, thereby resulting in increases in the stability of existing mRNAs. Here, we treated 6-week-old mdx mice for 4 weeks with the clinically-used anticoagulant drug heparin known to activate p38 and determined the impact of this pharmacological intervention on the dystrophic phenotype. Our results show that heparin treatment of mdx mice caused a significant ~1.5 to 3-fold increase in utrophin A expression in diaphragm, EDL and TA muscles. In agreement with these findings, heparin-treated diaphragm and TA muscle fibers showed an accumulation of utrophin A and β-dystroglycan along their sarcolemma and displayed improved morphology and structural integrity. Moreover, combinatorial drug treatment using both heparin and AICAR, the latter targeting AMPK and the transcriptional activation of utrophin A, caused an additive effect on utrophin A expression in dystrophic muscle. These findings establish that heparin is a relevant therapeutic agent for treating DMD and illustrate that combinatorial treatment of heparin with AICAR may serve as an effective strategy to further increase utrophin A expression in dystrophic muscle via activation of distinct signalling pathways.
Introduction

Duchenne Muscular Dystrophy (DMD) is the most common form of muscular dystrophy as it affects ~1 in 3500 male live births (Emery, 1991). Due to progressive muscle wasting, patients become wheelchair bound by early teens and death ensues in their second or third decade of life most often as a result of respiratory or heart failure (Anderson and Kunkel, 1992; Baxter, 2006; Bushby et al., 2010; Gayraud et al., 2010; Ryan et al., 2014). DMD is an X-linked recessive disorder caused by mutations/deletions in the dystrophin gene that prevent the production of functional dystrophin protein at the sarcolemma of individual muscle fibers (Nigro and Piluso, 2015; Straathof et al., 2015; Worton and Thompson, 1988). In these fibers, dystrophin plays a key role in maintaining their structural integrity by linking cytoplasmic actin filaments to the extracellular matrix via a multifunctional signaling complex called dystrophin-associated protein complex (DAPC) (Ehmsen et al., 2002; Johnson et al., 2013; Strakova et al., 2014; Yang et al., 1995). A lack of dystrophin protein as seen in DMD muscle fibers, induces cycles of muscle degeneration and regeneration with an eventual failure of the regenerative capacity thereby leading to a loss of muscle mass and function. Several therapeutic interventions are currently being developed for treating DMD including suppression of premature termination codons (Barton-Davis et al., 1999; Malik et al., 2010), exon skipping (Goyenvalle et al., 2004, 2015; Lu et al., 2005; Yokota et al., 2009, 2012) and gene-based therapies (Gregorevic et al., 2004, 2008; Rodino-Klapac et al., 2013).

An alternative strategy for treating DMD is focused on increasing the endogenous levels of the autosomal homolog of dystrophin, named utrophin (Blake et al., 2002; Love et al., 1989), in order to compensate functionally for the lack of dystrophin. Unlike dystrophin, which is expressed along the entire length of the sarcolemma in adult muscle fibers, expression of utrophin A (the
muscle isoform) is mainly restricted to neuromuscular and myotendinous junctions (Khurana et al., 1991; Ohlendieck et al., 1991). In addition to differences in their localization, dystrophin and utrophin A also differ in their ability to bind microtubules and in their ability to localize neuronal nitric oxide synthase (nNOS) at the sarcolemma (Belanto et al., 2014). However, similar to dystrophin, utrophin A associates with members of DAPC (Ishikawa-Sakurai et al., 2004; Peters et al., 1998) and with cytoskeletal F-actin filaments (Perkins and Davies, 2002; Winder et al., 1995) and can thus serve to provide functional integrity to individual muscle fibers. As an eventual effective therapeutic strategy, expression of utrophin A needs to extend from synaptic and myotendinous sites well into extrasynaptic regions, all along the sarcolemma of dystrophic fibers. Interestingly, utrophin A expression is naturally upregulated in dystrophic muscles of mdx mice and DMD patients. However, this upregulation is not sufficient to compensate for the loss of dystrophin in these dystrophic muscles (Kleopa et al., 2006; De la Porte et al., 1999; Mizuno et al., 1993). In this context, several studies have shown that transgenic overexpression of utrophin A in skeletal muscle of mdx mice alleviates the dystrophic phenotype (Krag et al., 2004; Perkins et al., 2001; Rafael et al., 1998; Squire et al., 2002; Tinsley et al., 1996). As a therapeutic strategy, utrophin A upregulation offers the distinct advantage that it would be beneficial for all Duchenne and Becker patients since this approach is independent of the exact nature of the patient’s mutation.

Given the therapeutic potential of increasing expression of utrophin A in dystrophic muscle, many laboratories around the world are devoting considerable efforts to identify drugs/small molecules that can stimulate utrophin A expression. One approach consists in screening libraries of chemical compounds in attempts to identify such active drugs/small molecules. To date, one such compound, i.e. SMT C1100, was shown to upregulate utrophin A in various experimental models and has since moved into clinical trials (Khurana and Davies, 2003; Moorwood et al., 2011,
2012; Tinsley et al., 2015). Alternatively, the characterization of molecular events and signalling pathways represents an attractive route to identify pharmacologically-active molecules that can act upon these pathways. In this context, several transcriptional regulatory mechanisms have been characterized including those that involve MyoD/myogenin and an E-box (Gramolini and Jasmin, 1999), GA-binding protein (GABP) and an N-Box (Dennis et al., 1996; Gramolini et al., 1997), calcineurin/Nuclear factor of activated T-cells (NFAT) signalling (Angus et al., 2005; Chakkalakal et al., 2003), peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) (Ehrenborg and Krook, 2009; Luquet et al., 2003; Wang et al., 2004), peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (Lin et al., 2002; Lira et al., 2010; Ljubicic et al., 2011), sirtuin 1 (Sirt1) (Chalkiadaki et al., 2014), and Ets-2 repressor factor (ERF) and E2F1 (Blanchet et al., 2011; Perkins et al., 2007). Collectively, these studies have led to the idea that promotion of a slower, more oxidative phenotype, which concomitantly upregulates utrophin A, is a viable therapeutic strategy for DMD (Al-Rewashdy et al., 2014; Chakkalakal et al., 2003; Chalkiadaki et al., 2014; Ljubicic et al., 2011, 2014; Miura et al., 2009). Over the last few years, several studies have used specific drugs to activate these various pathways in order to explore their therapeutic potential in mdx mice. In particular, treatment of mdx mice with either GW501516, 5-amino-4-imidazolecarboxamide riboside (AICAR)/metformin and resveratrol, which respectively target PPARβ/δ, 5′ adenosine monophosphate-activated protein kinase (AMPK), and Sirt1 and hence act primarily at the transcriptional level, results in an upregulation of utrophin A in muscle and functional benefits (Boon et al., 2008; Jørgensen et al., 2007; Ljubicic and Jasmin, 2015; Ljubicic et al., 2011, 2014; Miura et al., 2009; Olson et al., 2012; Riserus et al., 2008; Winder et al., 2000).

In addition to transcriptional regulatory mechanisms, earlier work from our laboratory has also shown the important contribution of post-transcriptional events in controlling utrophin A
expression. In fact, the 3’UTR and 5’UTR of utrophin A have both been shown to play key roles in the post-transcriptional regulation of utrophin A by regulating the stability and translation of utrophin A mRNAs, respectively (Chakkalakal et al., 2008; Gramolini et al., 2001; Miura et al., 2005, 2008, 2010; Moorwood and Khurana, 2013). Of particular pharmacological and therapeutic relevance, we demonstrated that the 3’UTR of utrophin A transcripts contains the cis-acting element ARE (AU-rich element) (Chakkalakal et al., 2008; Gramolini et al., 2001), known to be important for post-transcriptionally regulating mRNA stability (Apponi et al., 2011; Bakheet et al., 2003; Basu et al., 2011). In this context, we also showed recently that activation of p38 MAP kinase signalling in muscle reduces the binding of the destabilizing RNA-binding protein called K-homology splicing regulatory protein (KSRP), to the ARE which in turn, causes an increase in the stability of pre-synthesized utrophin A transcripts (Amirouche et al., 2013). In this latter study, we also noted that a short, 10-day regimen of heparin injections, which is currently used in clinical settings as an anticoagulant and also known to activate p38, induced a marked increase in utrophin A expression in the diaphragm muscle of mdx mice (Amirouche et al., 2013). These results, therefore, represent proof-of-principle that drugs that modulate post-transcriptional pathways can promote utrophin A expression in muscle. What remains unclear however, is whether a longer heparin treatment such as 4-week treatment periods used in recent pre-clinical studies (Amirouche et al., 2013; Jørgensen et al., 2007; Ljubicic et al., 2011; Zbinden-Foncea et al., 2012), can sufficiently increase utrophin A levels to provide morphological and functional benefits to muscle cells.

The aim of the current study was two-fold. First, we examined the impact of treating mdx mice for 4 weeks with heparin on expression of utrophin A in a variety of muscles and determined
its beneficial effects on the morphology and integrity of muscle fibers. Our second objective was to investigate the effect of combining two different drugs, namely heparin and AICAR, on utrophin A expression to determine whether they could instigate an additive effect on utrophin A levels in mdx mouse muscle. In this second series of experiments, we hypothesized that activating distinct yet, complementary pathways (AICAR acting transcriptionally via AMPK and PGC-1α, and heparin acting post-transcriptionally via KSRP and the ARE) would overcome the issue of saturating common downstream targets with multiple drugs/small molecules, thereby promoting additive effects on utrophin A expression in muscle.
Results

Heparin Stimulates Utrophin A Expression in Cultured Muscle Cells.

Heparin, a naturally occurring polysaccharide, is one of the oldest drugs used as an anticoagulant for treatment of thrombosis (Gray et al., 2008). Heparin has been shown to activate p38 MAPK activity in skeletal muscle of wild-type mice (Zbinden-Foncea et al., 2012). Our laboratory was first to demonstrate that short-term pharmacological activation of p38 by heparin, stimulates utrophin A expression in C2C12 muscle cells and mdx mouse muscle through a post-transcriptional mechanism involving inhibition of KSRP and enhanced stability of existing utrophin A mRNAs (Amirouche et al., 2013). In the present study, we first decided to extend these findings by examining whether a 4-week treatment with heparin upregulates utrophin A expression in several mdx mouse muscles and provides morphological and functional benefits to dystrophic muscles.

Initially, we treated C2C12 myoblasts for 24 hours with 2.5 IU/mL of heparin and subsequently harvested the cells and analyzed utrophin A expression using western blot and RT-qPCR. Heparin treatment induced a significant ~2-fold (P < 0.05) upregulation of utrophin A in C2C12 muscle cells compared to vehicle-treated cells (Figure 7A, B). Moreover, treatment of C2C12 myoblasts with heparin also caused a significant increase (P < 0.05) in utrophin A mRNA levels (Figure 7C). Finally, heparin treatment of cells transfected with a luciferase reporter construct containing the full-length 3’UTR of utrophin A, resulted in a ~1.5-fold increase (P < 0.05) in luciferase activity compared to vehicle-treated control cells (Figure 7D). In these latter experiments, we verified that heparin had no direct effect on the luciferase reporter activity per se by measuring the activity of the luciferase construct without the utrophin A 3’UTR in cells treated with or without heparin (luciferase activity (%) : luciferase+vehicle = 100, luciferase +heparin = 107, ± 2.49 (mean and SD). Together, these data demonstrate that heparin treatment enhances
Figure 7. Heparin increases utrophin A levels in C2C12 muscle cells. A) Representative western blot of utrophin A and ponceau staining using protein extracts from C2C12 myoblasts treated with vehicle control (water) or heparin (2.5 IU/ml) for 24 hours. B) Quantification of utrophin A protein levels as shown in (A). C) Utrophin A mRNA levels as measured by RT-qPCR using total RNA from C2C12 myoblasts treated with either vehicle (water) or heparin (2.5 IU/ml) for 24 hours. The values are normalized to 18S mRNA levels and graphed as a percentage to control. D) The activity of the luciferase reporter construct containing the full-length 3’UTR of utrophin A mRNA transfected into C2C12 myoblasts treated with either vehicle (water) or heparin (2.5 IU/ml) for 24 hours. N = 3 (with 3 replicates each). Error bars represent SEM. *P <0.05 versus vehicle control.
utrophin A expression in C2C12 muscle cells via a post-transcriptional mechanism thereby corroborating our earlier findings (Amirouche et al., 2013).

**Heparin Stimulates Utrophin A Expression in Several mdx Mouse Muscles.**

Based on these results, we progressed to treat 6-week old dystrophin-null, mdx mice with daily subcutaneous injections of heparin at 500 IU/kg for 4 weeks, which extends the duration of the short-term, 10-day treatment protocol used in our recent study (Amirouche et al., 2013). Here, we chose 4 weeks as a treatment duration since several pre-clinical studies from other labs using mdx mice (Jahnke et al., 2012; Tinsley et al., 2011), and ours (Ljubicic et al., 2011; Miura et al., 2009), have previously also used such a time period to test the efficacy of various compounds thus allowing for appropriate comparison of results.

As illustrated in Figure 8, 4 weeks of heparin treatment significantly increased (P < 0.05) utrophin A protein levels in diaphragm and tibialis anterior (TA) muscles of mdx mice (Figure 8A, B). Such an increase in utrophin A protein expression was also observed in the extensor digitorum longus (EDL) muscle (Figure 8C). Remarkably, the increased expression of utrophin A in the fast TA and EDL muscles was greater than ~ 3.5- and 2-fold, respectively. Nonetheless, there was no significant increase (P > 0.05) in utrophin A expression in the soleus most likely because utrophin A levels are already elevated in this slow oxidative muscle compared to fast glycolytic muscles (Gramolini et al., 2001a) (Figure 8D). Immunofluorescence experiments further established that heparin treatment indeed caused an increase in utrophin A levels in mdx mouse muscles, while also showing that this increase occurred at the sarcolemma of both diaphragm and TA muscle fibers (Figure 9A). Additionally, we assessed the localization of β-dystroglycan, a member of the DAPC, in order to examine whether heparin treatment caused reassembly of the DAPC along the
Figure 8. Heparin treatment induces upregulation of utrophin A protein levels in diaphragm, TA and EDL muscles from mdx mice. 6 week-old mdx mice were treated with heparin (500 IU/kg) or with vehicle (saline) for 4 weeks. Representative western blots and quantification of utrophin A protein levels standardized to ponceau using protein extracts from A) diaphragm (DIA), B) TA, C) EDL and D) soleus muscles. N = 4. Error bars represent SEM, *P < 0.05 versus mdx vehicle control. Note the significant ~1.5- to ~3-fold increase of utrophin A protein levels in diaphragm, TA and EDL muscles.
sarcolemma. Similar to what we observed with utrophin A by immunofluorescence, heparin treatment enhanced β-dystroglycan expression along the sarcolemma of fibers from the diaphragm and TA muscles of mdx mice (Figure 9B). Our data collectively show that a 4 week-treatment with heparin stimulates utrophin A expression in several mdx mouse muscles and induces its accumulation at the sarcolemma of dystrophic fibers together with that of an important DAPC component.

**Heparin Treatment Improves Morphological Properties of mdx Muscle.**

Given the findings shown above indicating assembly of utrophin A-containing DAPC at the sarcolemma, which implies an increase in the stability and integrity of dystrophic myofibers, we performed a series of haematoxylin and eosin (H and E) staining on cryostat sections of diaphragm and TA muscles from heparin- and vehicle-treated mdx mice. First, we measured the extent of central nucleation in these muscles. Central nucleation serves as a key indicator of muscle damage and regeneration in dystrophic muscle fibers (Briguet et al., 2004). Our results revealed that heparin treatment caused a significant (P < 0.05) decrease in the percentage of central nucleation in both diaphragm and TA muscles of treated mdx mice as compared to control mdx mice (Figure 10 A, D). Since mdx muscle fibers go through cycles of muscle degeneration and regeneration, a decrease in central nucleation is an indication of reduced degeneration (Briguet et al., 2004).

To determine if heparin treatment provided additional morphological benefits to mdx muscle fibers, we also assessed the fiber size distribution by measuring the cross-sectional area (CSA) of individual fibers from diaphragm and TA muscles obtained from heparin- versus vehicle-treated mdx mice. Histopathological studies have shown that abnormal fiber size distribution is a hallmark
Figure 9. Heparin increases sarcolemmal localization of utrophin A and promotes reassembly of the dystrophin-associated protein complex (DAPC) in mdx muscle fibers. A) Representative examples of cross-sections obtained from diaphragm and TA muscles of wildtype (WT) mice and mdx mice treated with heparin or vehicle (saline), were immunostained with utrophin A (UTR-A) antibody. B) Similar cross-sections from diaphragm and TA muscles were also immunostained with a β-dystroglycan (β-dys) antibody showing reassembly of the DAPC at the sarcolemma. Scale bar = 50 μm.
Figure 10. Heparin treatment improves morphological features of mdx muscle fibers. A) Representative examples of cross-sections of diaphragm (DIA) and TA muscles from wildtype mice (WT) and mdx mice treated with heparin or with vehicle (saline) that were stained using hematoxylin and eosin. Black arrows indicate the position of nuclei (centrally nucleated or at the periphery of fibers). B) Graphical summary of cross-sectional area (CSA) (*P < 0.05, **P < 0.01, ***P < 0.001, significantly different from mdx vehicle control). Median CSAs of each muscle are displayed above the frequency histograms. C) Variance coefficient measurements of the TA and diaphragm muscle fibers. D) Percentage of central nucleation in TA and diaphragm muscle fibers. N = 4. Error bars represent SEM, *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from respective control (WT or mdx vehicle). Scale bar = 50 μm.
of dystrophic muscles (Briguet et al., 2004; Torres and Duchén, 1987) since, for example, mdx mice display an abnormal proportion of small and large muscle fibers (Briguet et al., 2004). Our analyses demonstrate that the CSA profiles of vehicle-treated mdx mice display large differences when compared to healthy wild-type mice (Figure 10A, B). Four weeks of heparin treatment of mdx mice caused a rightward shift and normalization in the CSA frequency distribution of both diaphragm and TA muscles towards wild-type values with some fibers showing signs of hypertrophy. The reason why some of the mdx muscle fibers treated with heparin became hypertrophic is unclear. However, if toll-like receptors (TLR) are activated by heparin as suggested previously (Zbinden-Foncea et al., 2012), it may be that activation of these receptors by heparin induces IL-6 production (Machida et al., 2006) which could promote muscle fiber hypertrophy (Pillon et al., 2013; Serrano et al., 2008).

To complement these data, we also calculated the variance coefficient (VC) based on individual CSA of muscle fibers. As illustrated in Figure 10C, our results show that diaphragm and TA fibers from vehicle-treated mdx mice display significantly (P < 0.001) higher VC as compared to wild-type mice. Moreover, heparin treatment caused a reduction of the VC in both diaphragm (P < 0.001) and TA (P < 0.05) muscles from treated mdx mice. Together, these data indicate that 4 weeks of heparin causes important improvements in the morphology of dystrophic muscle fibers.

In addition to this morphological analysis, we also performed a fiber typing study to determine whether heparin treatment leads to a change in the expression of the slow, oxidative versus fast, glycolytic myofiber program. In comparison to fast-twitch muscles, slow, oxidative fibers express considerably more utrophin A and show reduced dystrophic damage (Gramolini et al., 2001a). Moreover, our recent work and that of others (Al-Rewashdy et al., 2014; Gordon et
al., 2013; Ljubicic et al., 2011; Miura et al., 2009), had led to the idea that promotion of the slow oxidative phenotype is particularly beneficial to mdx mouse muscle since such a fiber type transition is accompanied by increases in utrophin A expression.

Immunofluorescence experiments using myosin heavy chain (MHC) antibodies showed that 4 weeks of heparin treatment of mdx mice caused a significant increase (P < 0.001) in the number of MHC type I fibers in TA muscles compared to vehicle-treated mdx mice (Figure 11A, B). Moreover, western blotting revealed that expression of the oxidative phosphorylation (OXPHOS) markers, complex proteins IV and V were significantly (P < 0.05) elevated in TA muscles from heparin-treated mdx mice (Figure 11C, D). These findings suggest that 4 weeks of heparin treatment induced an increase in the expression of the slow oxidative myogenic program in mdx mouse muscle.

**Heparin Enhances the Sarcolemmal Integrity of mdx Muscle Fibers.**

To functionally examine the beneficial impact of heparin treatment on the structural integrity of mdx myofibers, we conducted IgM immunostaining on diaphragm and TA muscle cryosections. IgM is typically an extracellular protein that can penetrate within the muscle fiber when the sarcolemmal integrity is compromised as seen in mdx myofibers (Miura et al., 2009; Straub et al., 1997). Detailed examination of the staining showed that IgM penetration within fibers was markedly reduced in both diaphragm and TA muscles from heparin- versus vehicle-treated mdx mice (Figure 12A).

In a final series of experiments, we further assessed sarcolemmal integrity by employing an *ex vivo* protocol of repetitive eccentric contractions with EDL muscles as used in an earlier
Figure 11. Heparin treatment promotes expression of the slow, oxidative myogenic program in mdx mouse muscle. A) Immunofluorescence of TA muscle cross-sections was performed to detect myosin heavy chain type I (MHC I) in wildtype mice (WT) and mdx mice treated with heparin or vehicle. B) Graphical summary of the percentage of MHC I-positive in TA muscles. C and D) Representative western blots and quantitative analyses of the expression of OXPHOS complex IV subunit I, and OXPHOS complex V. Note the significant increase of MHC I-positive fibers (indicating a slow phenotype) and upregulation of the oxidative markers in TA muscles from heparin-treated mdx mice. N = 4. Scale bar = 50 μm. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001, (significantly different from mdx vehicle control).
study (Petrof et al., 1993) and in ours (Al-Rewashdy et al., 2014; Ljubicic et al., 2011; Miura et al., 2009). When determining maximal force, we observed that EDL muscles from heparin-treated mdx mice showed a trend toward normalization since force output was similar to wild-type mice (P > 0.05) and higher than vehicle-treated mdx mice (P = 0.1; Figure 12B). During repetitive eccentric contractions, reduction of force over time is known to correlate with damage of mdx EDL myofibers (Al-Rewashdy et al., 2014; Miura et al., 2009; Petrof et al., 1993). In our analysis, eccentric force measurements over time showed no difference (P > 0.05) between EDL muscles from heparin- and vehicle-treated mdx mice (data not shown). Such a discrepancy between force production and resistance to eccentric contractions has been observed in previous pre-clinical studies with the mdx mouse (Bogdanovich et al., 2002), including ours using AICAR treatment (Ljubicic et al., 2011). During this eccentric contraction protocol, EDL muscles are bathed in 0.1% trypan blue staining solution which can penetrate into the cytoplasm of myofibers in response to contraction-induced muscle damage. Examination of the percentage of trypan blue-stained EDL muscle fibers revealed that heparin caused a ~1.4-fold decrease (P < 0.01) in mdx mice versus vehicle-treated animals (Figure 12C). Collectively, these results show that 4 weeks of heparin induced an increase in utrophin A expression in several mdx mouse muscles together with multiple morphological and functional benefits.

**Combinatorial Treatment with AICAR and Heparin Causes an Additive Effect on Utrophin A Expression.**

AICAR, a synthetic agonist, has been shown to stimulate AMPK activity in skeletal muscle (Merrill et al., 1997) and recent work from our laboratory (Al-Rewashdy et al., 2014; Ljubicic et al., 2011) and that of others (Jahnke et al., 2012), demonstrated that a 4-week AICAR treatment
Figure 12. Heparin improves functional parameters of mdx mouse muscle. A) Representative examples of cross-sections of diaphragm (DIA) and TA muscles from wildtype (WT) mice and mdx mice treated with heparin or vehicle, that were immunostained with goat-anti mouse IgM Alexa 594. B) Shows the maximal force output of EDL muscles from WT mice and mdx mice treated with heparin or vehicle, obtained during the repetitive eccentric contraction protocol and generated at 200 Hz. C) Quantitative analysis of trypan blue staining of muscle fibers (% of total fibers). N = 4. Error bars represent SEM, *P < 0.05, **P < 0.01 versus respective control (WT or mdx vehicle). Scale bar = 50 μm.
of mdx mice resulted in utrophin A upregulation in muscles as well as in an attenuation of the dystrophic phenotype. Thus, given the effective upregulation of utrophin A in response to individual AICAR and heparin treatments, we set out to determine whether a combinatorial treatment of AICAR plus heparin (A+H) causes greater increases (additive or synergistic) in utrophin A expression. Such a combinatorial A+H treatment is expected, based on recent findings (Amirouche et al., 2013; Holmes et al., 1999; Irrcher et al., 2008; Jørgensen et al., 2007; Ljubicic et al., 2011; Zbinden-Foncea et al., 2012), to activate distinct signaling pathways acting transcriptionally (AICAR) and post-transcriptionally (heparin). To this end, we first treated C2C12 myoblasts with AICAR, heparin and a combinatorial A+H treatment for 24 hrs and examined utrophin A mRNA expression by RT-qPCR. As shown in Figure 13A, the combinatorial A+H treatment triggered an additive effect on utrophin A expression since it caused a ~ 2.5-fold increase (P < 0.05) in its mRNA level (compared to vehicle), which was also significantly higher (P < 0.05) than the effect seen with individual AICAR or heparin treatments.

Given these positive findings obtained with cultured cells, we proceeded to test whether the combinatorial A+H treatment further stimulates utrophin A expression in mdx mouse muscles as compared to individual treatments. To achieve this, we treated 6-week old mdx mice with subcutaneous injections of saline (control), AICAR (500 mg/kg body weight), heparin (500 IU/kg) and A+H for a duration of 4 weeks. The treatment and dosage protocols selected for these experiments were based on prior studies from our laboratory (Amirouche et al., 2013; Ljubicic et al., 2011) and others (Bamford et al., 2003; Buhl et al., 2002; Drake et al., 2010; Fillmore et al., 2010; Jørgensen et al., 2007; Leick et al., 2010; Zbinden-Foncea et al., 2012). We first measured utrophin A mRNA levels in diaphragm and TA muscles from treated mdx mice. Our results showed that utrophin A mRNA levels were significantly (P < 0.05) stimulated in response to all
Figure 13. Combinatorial treatment of mdx mice with AICAR and heparin increases utrophin A mRNA and protein expression. A) C2C12 cells were treated with AICAR (1 mM), heparin (2.5 IU/ml), AICAR + heparin, or vehicle for 24 hours. Utrophin A mRNA levels in treated C2C12 cells were measured by RT-qPCR and standardized to 18S mRNA levels. B) Utrophin A mRNA levels in diaphragm (DIA) and TA from mdx mice treated with AICAR (500 mg/kg), heparin (500 IU/kg), AICAR + heparin or vehicle for 4 weeks were measured by RT-qPCR. Values were standardized to 18S mRNA levels. C) Representative western blots of utrophin A and ponceau stain of protein extracts from diaphragm and TA muscles from vehicle or treated mdx mice. D) Quantification of utrophin A protein levels standardized to ponceau as shown in (C). N = 4 (3 replicates for each for RT-qPCR cell culture experiments). Error bars represent SEM. *P < 0.05 versus C2C12 cells + vehicle or mdx + vehicle, and $ = P < 0.05$ different compared to all treatments and vehicle.
three treatments (AICAR, heparin and A+H) in the diaphragm as compared to vehicle-treated mdx mice (Figure 13B). Although the combinatorial treatment did not show a statistically significant greater increase in utrophin A mRNA expression, there was nonetheless a clear trend for an additive effect in comparison to individual AICAR and heparin treatments. In TA muscles, no significant difference in utrophin A mRNA levels of treated mdx mice was detected but there was a clear trend towards an increase in utrophin A transcripts with the combinatorial treatment (Figure 13B).

We also measured utrophin A protein levels in both diaphragm and TA muscles of mdx mice treated with AICAR, heparin and A+H. In this case, and as seen with cultured myogenic cells, the combinatorial A+H treatment clearly resulted in a ~ 2.9-fold additive effect on utrophin A protein expression (P < 0.05) in the diaphragm of mdx mice compared to vehicle-treated mdx mice. This increase was also significantly greater (P < 0.05) than the increase seen with AICAR and heparin alone (Figure 13C, D). Similarly, all treatments induced an upregulation of utrophin A in the TA muscle with the combinatorial A+H treatment showing again an additive effect with a ~ 2.3-fold increase (Figure 13C, D).

Since AICAR is associated with heart hypertrophy and that p38 MAPK signaling has also been reported to be connected to cardiac failure and hypertrophy (Buhl et al., 2002; Clerk and Sugden, 2006; Liang and Molkentin, 2003; Liao et al., 2001; Streicher et al., 2010), we also examined whether there was an increase in utrophin A expression in the heart following the various drug treatments. Our results show that individual treatments of mdx mice with heparin or AICAR did not increase utrophin A levels in the heart (Figure 14). By contrast, the combinatorial therapy caused a more modest but significant increase in utrophin A expression (P < 0.05; Figure 14A, B). In these experiments, we also noted that utrophin A in the mdx heart is relatively high compared
Figure 14. Combinatorial treatment of mdx mice with AICAR and heparin increases utrophin A expression in the heart. A) Representative western blot of utrophin A and ponceau stain of protein extracts from heart muscle from treated mdx mice with AICAR (500 mg/kg), heparin (500 IU/kg), AICAR + heparin or vehicle for 4 weeks. B) Quantification of utrophin A protein levels standardized to ponceau as shown in (A). C) Representative western blot of utrophin A and ponceau stain of protein extracts from diaphragm, TA and heart muscle from vehicle-treated mdx mice. D) Quantification of utrophin A protein levels standardized to ponceau as shown in (C). N = 4. Error bars represent SEM. *P < 0.05 versus mdx + vehicle.
to TA and diaphragm muscles (Figure 14C, D) which, similar to the results obtained with the soleus (Figure 8), suggests that muscles with endogenously high utrophin A levels may not respond robustly to individual heparin or AICAR treatments.

Next, we performed immunofluorescence experiments on both diaphragm and TA muscles to establish if the combinatorial A+H treatment stimulated utrophin A expression along the sarcolemma of treated mdx mice. As shown in Figure 15, utrophin A expression was enhanced in both muscles in response to all three treatments (AICAR, heparin, A+H) but, consistent with the western blotting data, the increase appeared even greater in the diaphragm and TA muscle from the A+H group of mdx mice. To determine if the combinatorial A+H treatment leads to the reassembly of the DAPC complex, we also examined the localization of β-dystroglycan at the sarcolemma of mdx muscle fibers. As shown in Figure 15B, immunofluorescence experiments revealed that all treatments, including the combinatorial one with AICAR and heparin, enhanced β-dystroglycan expression along the sarcolemmal membrane in both diaphragm and TA muscles of treated mdx mice. Overall, these results indicate that in both cultured myogenic cells and mdx mouse muscles, the combinatorial A+H treatment is capable of inducing an additive effect on utrophin A expression as compared to individual treatments. The further increase in utrophin A expression seen following the combinatorial treatment, together with its localization at the sarcolemma and that of β-dystroglycan, suggest additional morphological and functional benefits over AICAR and heparin treatment alone.

**AICAR and Heparin Treatments Alters Expression of Key Signaling Molecules in mdx Mouse Muscles.**

As mentioned above, we showed in our recent work that AICAR promotes utrophin A
Figure 15. Combinatorial treatment of mdx mice with AICAR and heparin increases sarcolemmal localization of utrophin A in mdx muscle fibers. Representative cross-sections of diaphragm (DIA) and TA muscles from mdx mice treated with AICAR, heparin, AICAR + heparin or vehicle that were immunostained with utrophin A (UTR-A) A) and β-dystroglycan (β-dys) B) antibodies. Scale bar = 50 μm.
expression via a transcriptional mechanism involving PGC-1α (Al-Rewashdy et al., 2014; Ljubicic et al., 2011); [see also (Irrcher et al., 2003; Jahnke et al., 2012)] whereas heparin increases the stability of utrophin A mRNAs by decreasing the functional availability of KSRP (Amirouche et al., 2013). Here, we therefore verified that the combinatorial A+H treatment would similarly alter expression of these key molecules in treated mdx mice. First, we measured the expression levels of KSRP in muscles of mdx mice treated with AICAR, heparin and A+H. Our results show that heparin alone caused a ~ 2- and 2.5-fold decrease (P < 0.05) in KSRP protein levels in the diaphragm and TA muscles of mdx mice, respectively, as compared to vehicle-treated mdx mice (Figure 16A, B). Also, there was a trend towards a decrease in KSRP expression in both diaphragm and TA muscles following combinatorial A+H but this difference reached significance (P < 0.05) only in the diaphragm.

AICAR treatment is known to drive a significant increase in PGC-1α protein expression in mdx mouse muscle (Al-Rewashdy et al., 2014; Irrcher et al., 2003, 2008; Ljubicic et al., 2011; Suwa et al., 2003). Here, we observed that similarly, the combinatorial A+H treatment led to an increase (P < 0.05) in PGC-1α levels in both mdx diaphragm and TA muscles that is comparable, at least in the diaphragm, to that observed following the individual AICAR treatment (Figure 16C). Taken together, these findings suggest that the combinatorial A+H treatment regulates simultaneous expression of key and distinct signaling pathways that appear to ultimately converge onto the same slower, more oxidative phenotypic outcome in mdx mouse muscle which, in parallel, stimulate utrophin A expression.
Figure 16. Combinatorial treatment modulates expression of key signaling molecules in mdx mouse muscles. A) Representative western blots of KSRP protein levels in protein extracts from diaphragm (DIA) and TA muscles of mdx mice treated with AICAR, heparin, AICAR + heparin or vehicle. B) Quantification of KSRP protein levels standardized to β-actin as shown in (A). C) Representative western blots and quantification of PGC-1α protein levels standardized to β-actin from protein extracts of mdx diaphragm muscles. N = 4. Error bars represent SEM, *P < 0.05 versus mdx vehicle control.
Discussion

In the present study, we first determined whether a 4-week treatment regimen with heparin induces increases in utrophin A expression in several mdx mouse muscles and provides morphological and functional benefits. Our findings show that clinically-used heparin upregulates utrophin A levels in a variety of muscles of mdx mice including the diaphragm. This 4-week treatment also improved several pathological features of mdx mouse muscles including reassembly of the DAPC at the sarcolemma as well as improved morphological parameters and sarcolemmal integrity. As part of this work, we also explored the therapeutic potential of using a combinatorial treatment with both heparin and AICAR. Our results clearly show that combination of heparin with AICAR instigates an additive effect on utrophin A transcript and protein levels in mdx mouse muscle that is greater than increases seen when these drugs are used in isolation. Taken together, these results suggest that combinatorial treatment of heparin and AICAR, which individually target distinct signalling pathways, may serve as an effective therapeutic strategy to treat DMD.

Heparin Promotes the Post-transcriptional Upregulation of Utrophin A in Muscles and Improves the Dystrophic Pathology.

Initial evidence of possible post-transcriptional regulation of utrophin A came from earlier studies in which it was noted that altered expression of utrophin A protein in muscle was not mirrored by changes in levels of its mRNA (Gramolini and Jasmin, 1999; Pasquini et al., 1995b; Weir et al., 2002). Additional work revealed the importance of the 5’ and 3’UTRs of utrophin A mRNAs in regulating its translation and stability, respectively (Chakkalakal et al., 2008; Gramolini et al., 2001; Miura et al., 2008, 2010; Moorwood and Khurana, 2013). In this context, our laboratory showed that an ARE contained within the utrophin A 3’UTR plays a key role in
controlling the longevity of utrophin A mRNAs in muscle (Chakkalakal et al., 2008; Gramolini et al., 2001). Studies from our lab and others have shown that p38 MAPK regulates the levels of utrophin A mRNA (Amirouche et al., 2013) and other pre-synthesized transcripts (Briata et al., 2005), by decreasing the functional availability of KSRP; a RNA-binding protein that interacts with AREs and known to induce decay of target transcripts. More specifically, we reported in our initial study that p38 activation with heparin decreases the ability of KSRP to interact with the ARE present in the utrophin 3’UTR thereby causing an increase in utrophin A mRNA and protein levels (Amirouche et al., 2013).

Here, we show that heparin is a potent utrophin A stimulator since treatment with heparin causes a clear increase in utrophin A expression in both C2C12 muscle cells and in several mdx mouse muscles. Such increase in utrophin A expression in dystrophic muscles was accompanied by important morphological and functional improvements. A central question raised by these findings concerns the identity of the signaling molecules upstream of p38 activation on which heparin acts. Although the complete nature of this pathway remains unclear, converging lines of evidence indicate that toll-like receptors (TLR) may play a role in activating p38 MAPK (Senn, 2006; Zbinden-Foncea et al., 2012). TLRs are transmembrane proteins highly expressed in immune cells, however, recent studies have also found TLRs to be present in other cell types including TLR2 and 4 that are expressed in skeletal muscle cells (Rawat et al., 2010; Reyna et al., 2008; Senn, 2006). In mdx mice, TLR1, 2, 3, 4, 7, 8 and 9 are expressed in a variety of skeletal muscles at different levels. In fact, slow-twitch muscles, such as the soleus have more TLRs (Henriques-Pons et al., 2014). Of high relevance, heparin has been shown to activate p38 via TLR2 and TLR4 in C2C12 myotubes as well as skeletal muscle of C57BL/6J wild-type mice (Senn, 2006; Zbinden-Foncea et al., 2012). More specifically, heparin treatment results in increases in
extracellular levels of non-esterified fatty acid (NEFA) which in turn, directly activates the TLR receptors (Zbinden-Foncea et al., 2012). This suggests that heparin may stimulate TLR which then activates p38 MAPK causing a decrease in the functional availability of KSRP and in its binding to the ARE located within the utrophin 3’UTR, culminating in increases in utrophin A expression. Interestingly, levels of TLR4 mRNA have been shown to be increased in mdx mouse muscles compared to wildtype (Giordano et al., 2015), thus providing greater target availability for heparin in dystrophic muscle. However, recent work has also shown that ablation of TLR or of the TLR adaptor protein called myeloid differentiation primary response gene 88 (myd88) in mdx mice, offers benefits to their muscle (Giordano et al., 2015; Henriques-Pons et al., 2014). Therefore, it seems clear that additional work is necessary to better understand the role and therapeutic potential of TLR’s in dystrophic muscle.

**Heparin Promotes Expression of the Slow Oxidative Phenotype in Dystrophic Skeletal Muscles.**

Our laboratory was first to demonstrate that slower, more oxidative fibers have overall higher content of utrophin A in extrasynaptic regions as compared to fast glycolytic fibers (Gramolini et al., 2001a). Such increased expression of utrophin A correlates well with the fact that slow myofibers in both mdx mice and DMD patients show reduced damage in comparison to faster muscle fibers (Gramolini et al., 2001a). Accordingly, we speculated several years ago (Al-Rewashdy et al., 2014; Chakkalakal et al., 2003; Chalkiadaki et al., 2014; Ljubicic et al., 2011, 2014; Miura et al., 2009) that strategies to promote expression of the slow oxidative phenotype, which includes increased utrophin A expression, would be highly beneficial to dystrophic muscle. Using a variety of transgenic mouse models and pharmacological interventions, our lab (Al-Rewashdy et al., 2014; Ljubicic et al., 2011, 2014; Miura et al., 2009) and others (Chalkiadaki et
al., 2014; Ehrenborg and Krook, 2009; Gordon et al., 2013; Jørgensen et al., 2007; Luquet et al., 2003; Reyes et al., 2015; Riserus et al., 2008; Wang et al., 2004; Winder et al., 2000) have shown that indeed such an approach is therapeutically relevant as increased expression of the slow oxidative phenotype provides important morphological and functional benefits to dystrophic muscle. In this context, beneficial adaptations have been seen previously in mitochondria, the contractile apparatus and in the structural integrity of fast glycolytic muscle fibers following an increase in utrophin A and \( \beta \)-dystroglycan expression throughout the sarcolemma (Ljubicic et al., 2011; Marusich et al., 1997).

In the present study, we also show that treatment of mdx mice with heparin promotes a shift towards a slower, more oxidative phenotype. This suggests that the protective effects of heparin treatment on the progression of the disease may relate to its ability to stimulate the slow oxidative myogenic program. How heparin signals to achieve this transition in fiber type and metabolic profile is unclear at the present time but it would seem important to examine whether TLR’s play a role in such a signalling cascade. In agreement with our data, a recent study demonstrated that heparin induces mitochondrial membrane potential and increases expression of the slow oxidative marker cytochrome C oxidase subunit II in HUVECS cells (Wang et al., 2014). Thus, further studies are also warranted to determine if heparin, TLR’s and/or the downstream regulators p38 MAPK and KSRP, are key modulators of the slow oxidative phenotype.

Combinatorial Treatment of mdx Mice with Heparin and AICAR Induces an Additive Effect on Utrophin A Expression by Activating Distinct Pathways.

In the present study, we also decided to treat mdx mice with a combination of the putative post-transcriptional activator heparin (Amirouche et al., 2013; Zbinden-Foncea et al., 2012) with
AICAR, a mediator of utrophin A expression via transcriptional pathways (Ljubicic et al., 2011). In this work, we chose not to include an individual AICAR treatment in mdx mice because this has already been extensively studied in recent past by our lab (Al-Rewashdy et al., 2014; Ljubicic et al., 2011) and that of others (Bamford et al., 2003; Corton et al., 1995; Jahnke et al., 2012; Jørgensen et al., 2007; Leick et al., 2010). In these combinatorial experiments, we postulated that by activating distinct pathways, utrophin A levels would be further increased in comparison to treatments using these two drugs in isolation. Our results show that individual treatments of AICAR and heparin increase utrophin A but at a lower extent to that seen with the combination of both drugs. In fact, our combinatorial treatment with AICAR + heparin stimulated a clear and important additive effect on utrophin A expression in C2C12 muscle cells and mdx mouse muscle. More specifically, utrophin A protein levels were increased by a maximum of nearly 3-fold in response to the AICAR plus heparin treatment which is particularly encouraging since previous studies reported that a 2-fold increase in utrophin A in muscle is sufficient to ameliorate the dystrophic phenotype in mdx mice (Tinsley et al., 1998). The fact that the diaphragm responded well to the combinatorial treatment is important given that maintaining functions of respiratory muscles in DMD patients is central to efficacious therapeutic interventions.

Since utrophin A expression can be modulated by both transcriptional and post-transcriptional events, we also examined expression levels of utrophin A regulators following AICAR and heparin treatment. As predicted based on recent work (Fillmore et al., 2010; Ljubicic et al., 2011; Suwa et al., 2003), the individual treatment with AICAR and the combinatorial treatment of AICAR plus heparin resulted in significant increases in PGC-1α levels in dystrophic muscle; a change not observed following treatment with heparin alone. In contrast, individual treatment with heparin and the combinatorial treatment both promoted a decrease of KSRP
expression in treated mdx mouse muscle. However, the decrease of KSRP expression was greater following the individual heparin treatment compared to the combinatorial treatment and varied between TA and diaphragm muscles. The fact that utrophin A levels are nonetheless additively increased following the combinatorial treatment indicates that at least one other mechanism controls the functional availability of KSRP in muscle, in particular its sequestration by 14-3-3 as we have shown recently (Amirouche et al., 2013). Taken together, these results further highlight the distinct mechanisms of action of AICAR and heparin on utrophin A upregulation and indicate that the combination of these two drugs activates both pathways. On the basis of these findings, we therefore propose a model (Figure 17) by which AICAR and heparin stimulate utrophin A expression via distinct signalling cascades and molecular events. In this model, AICAR promotes utrophin A expression through activation of AMPK and PGC-1α signaling, whereas heparin acts mainly by activating p38 MAPK which subsequently phosphorylates KSRP and promotes its sequestration by 14-3-3 proteins while also decreasing its expression level. The combined effect of activating these pathways results in enhanced transcription of the utrophin gene with a parallel increase in the stability of synthesized utrophin A transcripts as illustrated in Figure 17.

One of the highlights of our study is the identification and exploitation of clinically-relevant drugs for treating DMD. Such a repurposing strategy exploits the off-target effects of clinically-approved drugs, thereby providing opportunities to accelerate the development of new drugs for DMD while reducing health risks (Ashburn and Thor, 2004). Previous studies from our laboratory exploited the off-target effects of several drugs in upregulating utrophin A expression in muscle such as the PPARβ/δ agonist GW501516, AMPK/Sirt1 activator Resveratrol, AMPK/PGC-1α activators Metformin and AICAR, as well as heparin (Amirouche et al., 2013; Ljubicic and Jasmin, 2015; Ljubicic et al., 2011, 2014; Miura et al., 2009). In our current study, it appears
Figure 17. Model depicting the potential signaling and impact on utrophin A expression of the combinatorial treatment. AMPK activation by AICAR activates PGC-1α expression in muscle. Upon coactivation, PGC-1α and transcription factors (TF) (for example PPARβ/δ and GABP) stimulate utrophin A transcription. P38 activation by heparin mediates a decrease in the functional availability of the RNA-binding protein KSRP, which is involved in ARE-mediated decay of utrophin A transcripts. This decrease in the functional availability of KSRP is achieved by: 1) an increase in its phosphorylation status which promotes its sequestration by the regulatory protein 14-3-3; and 2) a decrease in its expression. Simultaneous activation of these pathways by AICAR and heparin leads to an additive effect on utrophin A expression in dystrophic muscle.
that repurposing of AICAR and heparin may be an effective combinatorial therapeutic strategy to treat DMD. Heparin is an FDA approved drug that has been used to treat and prevent thrombosis for years (Gray et al., 2008). It is also a naturally occurring compound in the human body indicating that it is relatively safe with less severe side effects (Gray et al., 2008). On the other hand, AICAR is also being tested extensively in clinical trials for the treatment of other diseases such as type II diabetes (Babraj et al., 2009; Boon et al., 2008; Leick et al., 2010). Despite the beneficial effects of the combinatorial treatment of AICAR with heparin on utrophin A expression, it is important to be mindful of the limitations of this strategy in long-term therapies. AICAR and heparin are associated with a few side effects. In particular, heparin can cause excessive bleeding, induce thrombocytopenia, skin necrosis and osteoporosis in treated patients. Conversely, and due to its anticoagulant properties, heparin could also be further beneficial by increasing the effective supply of metabolites and oxygen to working muscle. AICAR, on the other hand, is associated with liver and heart hypertrophy (Buhl et al., 2002). In this case, metformin might represent an interesting alternative for activating AMPK (Ljubicic and Jasmin, 2015; Suwa et al., 2006). Nonetheless, one should remain well aware of these possible side effects and drug interactions, and ultimately balance the advantages versus disadvantages of using such drug therapies.
Materials and Methods

Cell Culture, Plasmid, Transfection and Luciferase assay.

Mouse C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were plated on 6-well culture dishes and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Wisent, St-Bruno, QC, Canada), 1% L-glutamine and 1% penicillin/streptomycin. The cells were incubated at 37°C with 5% CO₂ in a humidified chamber.

The full-length 3’UTR from the utrophin A mRNA (2.1 kb), which was used in these experiments, was described in a previous study (Amirouche et al., 2013). Briefly, the 3’UTR was isolated by RT-PCR and subcloned in the PGL4 vector backbone, downstream of the Firefly luciferase gene. Transient transfections were performed using the transfection reagent Lipofectamine (Invitrogen, Carlsbad, CA, USA) by following the manufacturer’s instructions. Approximately 50,000 cells per well were seeded in a 6-well culture dish. The next day, C2C12 cells were transiently transfected at 50-60% confluence with a mixture of DNA (1μg)/Lipofectamine for 4 hrs and incubated at 37°C in a humidified chamber supplied with 5% CO₂. Following the transfection, cells were treated for 24 hrs with heparin as described below. Cells were harvested 24 hrs later for further analyses.

C2C12 cells were homogenized in reporter lysis buffer (Dual Luciferase Assay System, Promega, Madison, WI, USA), followed by a freeze thaw treatment. The activity of Firefly luciferase was determined using the Dual Luciferase Assay kit and detected using a luminometer (Lumat LB 9507 - Berthold Technologies, Große Enz, Germany). Cells were co-transfected with a control Renilla luciferase reporter vector (phRGtk-luc) to monitor transfection efficiency.
**Heparin and AICAR Treatment.**

Approximately, 75,000 cells per well were seeded in a 6-well culture dish. The next day, C2C12 cells (60-70% confluence) were treated with heparin (2.5 IU/ml) (LEO Pharma, Thornhill, Canada), or/and AICAR (1mM) (TRC, Toronto, Canada), or sterile water (control) for 24 hrs as used in earlier studies (Amirouche et al., 2013; Irrcher et al., 2008). *In vivo* experiments were performed using mdx mice (The Jackson Laboratory, Bar Harbor, USA), that were maintained in the Animal Care and Veterinary Service of University of Ottawa under a constant 12 hr light-dark cycle with full access to water and food. The experimental protocols were approved by the University of Ottawa Animal Care Committee and were in accordance with the Canadian Council of Animal Care Guidelines. Six week-old mdx mice were treated daily with vehicle (sterile saline), AICAR (500 mg/kg), heparin (500 IU/kg) or AICAR + heparin by subcutaneous injections for 4 weeks (Amirouche et al., 2013; Jørgensen et al., 2007; Ljubicic et al., 2011; Zbinden-Foncea et al., 2012). Both AICAR and heparin were dissolved in a volume of 50 ml of sterile saline prior to daily treatments. Muscles were dissected from euthanized treated mdx mice, flash frozen in liquid nitrogen, or in melting isopentane cooled with liquid nitrogen, for further analyses.

**Ex-Vivo Eccentric Contractions.**

After 4 weeks, the heparin- and saline-treated mice were euthanized. The extensor digitorum longus (EDL) muscle was dissected and attached at one end to a Dual mode lever system (model 300C, Aurora Scientific, Aurora, Canada) to measure force and to lengthen muscle, and the other end to a fixed rod. Throughout the experiment, the muscle was submerged in a saline solution containing (in mM): 118.5 NaCl, 4.7 KCl, 2.4 CaCl$_2$, 3.1 MgCl$_2$, 25 NaHCO$_3$, 2 NaH$_2$PO$_4$, 5.5 D-glucose, 95% O$_2$-5% CO$_2$ (to maintain a pH of 7.4) and 0.1% trypan blue (Sigma-Aldrich,
Oakville, Canada), with a flow rate of 15 mL/minutes at 25°C. Adjustments of the muscle length were performed in order to get maximal force output. Five maximal tetanic contractions (400 ms train duration, 10 V, 0.3 ms square pulse, 200 Hz) were elicited to determine muscle contractile kinetics. These contractions were executed every 100s, followed by 12 eccentric contractions every 120 s (700 ms train duration, 10 V, 0.3 ms square pulse, 200 Hz) Eccentric contractions were elicited by subjecting muscles to a 10% lengthening at a velocity of 0.5 Le/s throughout the last 200 ms. Electrical stimulation were generated across two platinum wires (positioned above and below muscles 4 mm apart) using a Grass stimulator (model S88X, Grass Technologies, West Warwick, USA). A Keithley data acquisition board (model KPCI-3104, Cleveland, USA) was used to detect the force at a sample rate of 5KHz. Tetanic force was defined as the increase in force upon stimulation and was normalized to the cross sectional area (CSA). The muscle CSA was calculated by dividing muscle volume by the muscle experimental weight; muscle volume was calculated from the muscle weight converted to volume using a density of 1.06 g/cm³. The trypan blue-stained EDL muscles were frozen in melting isopentane cooled with liquid nitrogen and cut into 10 µm cross-sections. The sections were observed with a fluorescent Zeiss Axioshop-2 microscope. Stained fibers were quantified compared to total fiber using image J (NIH version 1.0).

**Western Blotting.**

Frozen muscles were ground to powder with a BioPulverizer on dry ice. Muscle samples were suspended in 300 uL of Urea extraction buffer (7 M UREA, 2 M Thiourea, 4 M CHAPS, 100 mM DTT, 125 mM Tris-HCl pH 6.8) supplemented with complete Mini Protease Inhibitor Cocktail and phosphatase inhibitor PhosSTOP (Roche, Laval, QC, Canada). The samples were
vortexed for 30 min at room temperature and then centrifuged at 20000g for 15 min. Supernatants were collected and stored at -80°C. Protein concentrations were determined using CB-X Protein Assay Kit (G-Biosciences, St. Louis, MO, USA) and bovine serum albumin was used as a standard. Ten μg of extracted proteins were loaded on a sodium dodecyl sulfate polyacrylamide gel (6-8% polyacrylamide) and migrated at 80-100V for 2-3 hours. The protein transfer was performed on a nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada). After transfer, membranes were stained with Ponceau S (Sigma-Aldrich, Oakville, Canada) to confirm equal loading between lanes. Membranes were subsequently washed 4 times with 1× PBS-T (1xPBS, 0.2% Tween) and blocked for 1 hr with a 5% skim milk in PBS-T solution. Blots were then incubated in blocking solution for 1 hr or overnight with antibodies directed against utrophin A (1:500; Novoceastra, Leica biosystems, Concord, ON, Canada), KSRP (1:1000; Bethyl Laboratories, Montgomery, TX, USA), PGC-1α (1:2000; abcam, Toronto, ON, Canada) or OXPHO cocktail (1:1000; abcam, Toronto, ON, Canada), with gentle rocking. The blots were incubated with appropriate Horse Radish Peroxidase-conjugated secondary antibody for 1 hr at room temperature in blocking solution and washed 4 times with 1xPBS-T. When appropriate, the blots were also incubated with antibodies against β-actin (1:10000; Santa Cruz, Dallas, Texas, USA) as a loading control. Ponceau staining was systematically used to verify equal loading for the utrophin A western blots. This was necessary to obtain better separation of large molecular mass proteins. The gels were run longer causing the lower mass proteins such as β-actin, GAPDH and tubulin to run out of the gels. We thus prefer staining the same membrane used for Western blotting for utrophin A in order to assess loading. Protein quantification of utrophin A was normalized to protein levels as determined from the same membrane stained with ponceau. The Chemiluminescent detection of proteins was
performed using ECL reagent (Perkin Elmer, Waltham, MA, USA). The films were quantified using ImageJ (NIH version 1.0) and/or Image Lab.

**Immunofluorescence, and Haematoxylin and Eosin Staining.**

Ten μm muscle cross-sections were processed for immunofluorescence using the M.O.M’s Immunodetection kit (Vector Laboratories, Burlington, ON, Canada). Sections were incubated with primary antibodies against utrophin A (NCL-DRP2) (1:200; Novocastra, Leica biosystems, Concord, ON, Canada), β-dystroglycan (1:400; NCL-B-DG, Novocastra, Leica biosystems, Concord, ON, Canada), or myosin heavy chain type 1 (undiluted; BA-F8) (Hybridoma Bank, Iowa city, IA, USA), for 30 min at room temperature. A Texas Red conjugated Streptavidin antibody (1:500; Vector laboratories, Burlington, ON, Canada) was used for detection. A FITC-conjugated IgM anti-mouse secondary antibody was used (1:400; Sigma-Aldrich, Oakville, Canada) for immunoglobulin (IgM) staining. The slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlington, ON, Canada) and visualized using a Zeiss Axioskop-2-fluorescence microscope. MHC positive fibers, compared to total fiber, were quantified using image J (NIH version 1.0).

Tibialis Anterior (TA) and diaphragm muscle cross-sections were also stained with hematoxylin and eosin dyes, dehydrated using a series of ethanol solutions (70%, 90%, 100%), and subsequently washed with toluene. The slides were then mounted using Permount and visualized using a Zeiss Axioskop-2 microscope. The percentage of central nucleation was determined by visually counting the total number of muscle fibers and the number of muscle fibers with central nucleation from 4-6 cross-sectional views using the Northern Eclipse Software (NES, Expix Imaging, Mississauga, Ontario, Canada). Cross-sectional Area (CSA) of each fiber was
measured using NES. The variance coefficient was calculated based on the CSA of muscle fibers using the formula “variance coefficient Z = 1000 x standard deviation of muscle fiber CSA/mean muscle fiber CSA”.

**RNA Extraction and RT-qPCR.**

Total RNA was extracted from muscle tissue and C2C12 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. TRIzol extracted RNA was treated for 1 hr with DNAse I (Invitrogen, Carlsbad, CA, USA) to eliminate possible DNA contamination. Reverse transcription (RT) was carried out using an RT reaction mixture containing 5 mM MgCl2, 1× PCR buffer, 1 mM dNTP, 1 U/ml RNase inhibitor, 5 U/ml Moloney murine leukemia virus reverse transcriptase and 2.5mM random hexamers (Applied Biosystems, CA, USA). A real-time quantitative PCR (qPCR) was performed on an MX3005p real-time PCR system (Stratagene, La Jolla, CA, USA) using a QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA, USA). For these experiments, amplification of the 18S ribosomal subunit, GAPDH, utrophin A was performed in duplicates with the following primer sequences: utrophin A - forward 5′-ATCTTGTGCGGGCTTTCAC-3′ and reverse 5′-ATCCAAAGGGCTTTCCAGAT-3′, 18S Ribosomal - forward 5′-CGCCGCTAGAGGTGAATC-3′ and reverse 5′-CCAGTCGGCATCGTTATGCC-3′, GAPDH - forward 5′-GGGTGTGAAACCACGAGA AAT-3′ and reverse 5′-CCTTCCCAATGCCAAAGTT-3′.

**Statistical Analysis.**

The data were analyzed using paired and unpaired student’s t-test and ANOVAs (Analysis of Variance) with Fisher’s post-hoc tests as appropriate. Error bars represent standard error of the
mean (SEM). Statistical analysis was done with StatView version 5.0 (SAS Institute Inc., Cary, NC, USA) on raw data prior to conversion to fold difference (compared to control). Significance was accepted at *$P \leq 0.05$.

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Chapter 3
Celecoxib treatment improves muscle function in mdx mice and increases utrophin A expression

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Author’s contribution:

Conceived and designed the experiments: CP, BJJ. Performed the experiments: CP, NA. Analyzed the data: CP, NA. Wrote the Manuscript: CP, BJJ. Contributions by figure: Fig 18 (CP, NA), Fig 19 (CP), Fig 20 (CP), Fig 21 (CP), Fig 22 (CP, NA), Fig 23 (CP, NA), Fig 24 (CP, NA), Fig 25 (NA)
Abstract

Duchenne muscular dystrophy (DMD) is a genetic and progressive neuromuscular disorder caused by mutations/deletions in the dystrophin gene. Although there is currently no cure, one promising therapy for DMD aims at increasing endogenous levels of utrophin A to functionally compensate for the lack of dystrophin. Recent studies from our lab revealed that heparin treatment of mdx mice activates p38 MAPK, leading to an upregulation of utrophin A expression and improvements in the dystrophic phenotype. Based on these findings, we sought to determine the effects of other potent p38 activators, including the COX-2 inhibitor celecoxib. Here, we treated 6-week-old mdx mice for 4 weeks with celecoxib. Immunofluorescence analysis of celecoxib-treated mdx muscles revealed a fiber type switch from a fast to a slower phenotype along with beneficial effects on muscle fiber integrity. In agreement, celecoxib-treated mdx mice showed improved muscle strength. Celecoxib treatment also induced increases in utrophin A expression ranging from ~1.5 to 2-fold in tibialis anterior diaphragm and heart muscles. Overall, these results highlight that activation of p38 in muscles can indeed lead to an attenuation of the dystrophic phenotype and reveal the potential role of celecoxib as a novel therapeutic agent for the treatment of DMD.

Key words: DMD, NSAID, skeletal muscle
Introduction

DMD is a devastating hereditary neuromuscular disease caused by mutations/deletions in the dystrophin gene. This childhood disorder is characterized by progressive muscle wasting and loss of ambulation before teenaged years, followed by respiratory and cardiac complications which ultimately lead to death in the third decade of the patient’s life (Bushby et al., 2010; Gayraud et al., 2010; Mosqueira et al., 2013; Ryan et al., 2014). In healthy muscle, dystrophin is a rod-shaped stabilizing protein that localizes at the sarcolemma of muscle fibers where it binds F-actin through its N-terminal domain and the dystrophin-associated glycoprotein complex (DAPC) through its C-terminus (Brenman et al., 1995; Ehmsen et al., 2002; Johnson et al., 2013; Koenig et al., 1988; Strakova et al., 2014; Yang et al., 1995). Loss of dystrophin as seen in DMD prevents formation of the DAPC at the sarcolemma thereby making muscle fibers more susceptible to damage. Such sarcolemmal damage results in excessive calcium infiltration, continuous cycles of degeneration and regeneration as well as increased inflammation, macrophage penetration, and the eventual replacement of muscle fibers by adipose and connective tissues (Giordano et al., 2015; Mojumdar et al., 2014; Nitahara-Kasahara et al., 2016; Villalta et al., 2009). Glucocorticoid treatment with prednisone or deflazacort is currently the only widely used therapy for DMD patients with some beneficial effects on symptom progression including delays of muscle fatigue, ambulation and scoliosis (DeSilva et al., 1987; Fenichel et al., 1991). Therefore, despite recent success with the exon skipping drug Exondys51 (eteplirsen), recently conditionally approved by the FDA (Irwin and Herink, 2017; Niks and Aartsma-Rus, 2017) and the progress in gene and cell therapies (Bogdanovich et al., 2004; Chakkalakal et al., 2005; Durbeej and Campbell, 2002; Goyenvalle et al., 2011; Gregorevic et al., 2004, 2008; Lu et al., 2011; Nakamura and Takeda, 2011; Rodino-
Klapac et al., 2013), there is still an urgent need to develop appropriate therapies with high efficiency, ease of administration, and wide applicability.

One possible approach for the treatment of DMD that could benefit all patients while also having systemic effects on affected muscles, consists of upregulation of the autosomal homolog of dystrophin named utrophin. Converging lines of evidence show that utrophin can serve as a substitute for dystrophin at the sarcolemma due to their high sequence identity and functional redundancy (Blake et al., 2002; Cohn and Campbell, 2000; Love et al., 1989). There are two utrophin isoforms: utrophin A, found predominantly in skeletal muscle fibers, brain and kidneys, and utrophin B, expressed mostly in the vascular endothelium (Baby et al., 2010; Weir et al., 2002). Several studies have shown that utrophin A preferentially localizes to the postsynaptic membrane of neuromuscular junctions in both healthy and dystrophic muscles. However, at distinct muscle developmental stages, or under specific conditions, utrophin A can also localize to extrasynaptic compartments of muscle fibers where it binds F-actin filaments and the DAPC at the sarcolemma in a manner analogous to dystrophin (Clerk et al., 1993; Ishikawa-Sakurai et al., 2004; Janghra et al., 2016; Perkins and Davies, 2002; Peters et al., 1998; Winder et al., 1995). In addition, transgenic overexpression of utrophin A in skeletal muscle of mdx mice has been shown to improve the dystrophic phenotype (Krag et al., 2004; Rafael et al., 1998; Squire et al., 2002; Tinsley et al., 1996). Thus, many laboratories worldwide, including our own, have focused their efforts on developing different strategies to upregulate utrophin A expression in dystrophic muscles. Such methods include increasing the recruitment of utrophin to the sarcolemma with biglycan (Amenta et al., 2011), and through stimulation of signaling pathways with active drugs/small molecules (Guiraud and Davies, 2017; Khurana and Davies, 2003; Moorwood et al., 2011, 2013; Tinsley et al., 2015). Ezutromid (SMT C1100) is one such compound that can upregulate utrophin A in
different experimental models (Guiraud et al., 2015b; Tinsley et al., 2011) and, importantly, recent results from Summit Therapeutics show that it can reduce muscle damage in DMD patients enrolled in clinical trials (Summit Therapeutics plc, 2018). Consequently, it is of great interest to identify novel drugs that modulate signaling pathways known to control expression of utrophin A in dystrophic muscles.

Recent studies from our lab revealed that a short 10-day treatment with the FDA-approved anticoagulant drug heparin activates p38 mitogen-activated protein kinase (MAPK) signaling, which caused downstream upregulation of utrophin A expression in diaphragm muscles of mdx mice and its localization to the sarcolemma (Amirouche et al., 2013). In addition, we have recently shown that a longer treatment of mdx mice with heparin indeed promotes upregulation of utrophin A in various muscles resulting in several functional, biochemical and histological adaptations that positively impact the dystrophic phenotype (Péladeau et al., 2016). Given these promising results obtained with heparin, we wondered whether other drugs known to activate p38 signaling could also induce beneficial effects on the dystrophic phenotype. In this context, one such FDA-approved drug known as celecoxib, and more commonly as Celebrex (Farooq et al., 2013; Hsiao et al., 2007; Steffel et al., 2006), appeared particularly attractive. This nonsteroidal anti-inflammatory drug (NSAID) is a specific cyclo-oxygenase-2 (COX-2) inhibitor commonly used as an anti-inflammatory treatment in many conditions such as osteoarthritis and rheumatoid arthritis. Previous work showed that celecoxib can activate p38 MAPK signaling in skeletal muscle cells (Hsiao et al., 2007; Steffel et al., 2006), and that it elicits beneficial effects in mouse models of Spinal Muscular Atrophy (SMA) (Farooq et al., 2013). In fact, several studies indicate that different NSAIDs may represent promising therapeutics for treating myopathies. For example, aspirin and ibuprofen treatments have been shown to improve muscle morphology, reduce immune
cell infiltration between muscle fibers, and ameliorate resistance to muscle fatigue (Miyatake et al., 2016; Serra et al., 2012). Additionally, the NSAID nabumetone was shown to upregulate endogenous utrophin mRNA and protein levels in C2C12 cultured muscle cells (Moorwood et al., 2011). Based on these findings, we thus decided to determine the impact on the dystrophic phenotype of a 4-week celecoxib treatment of mdx mice.
Results

Celecoxib enhances muscle strength of mdx mice and sarcolemmal integrity of dystrophic fibers.

In a first set of experiments, we investigated whether a 4-week treatment of 6-week old mdx mice with celecoxib (20µg/kg/day), could improve muscle strength. Accordingly, mice were subjected to a digital gauge containing a rectangular grid to detect overall grip strength. Throughout the treatment, we measured forelimb grip strength and weight of the mice. At the end of the treatment period, celecoxib markedly improved (normalized or unnormalized to body weight; P < 0.05 and P < 0.01) grip muscle strength of mdx mice compared to vehicle-treated mdx mice (Figure 18B). In fact, celecoxib-treated mdx mice had similar grip strength as wildtype controls. Moreover, normalization of grip strength measurements to mouse weight also showed the beneficial impact (P < 0.01) of celecoxib (Figure 18A, B).

After 4 weeks of treatment, mice were euthanized and skeletal muscles were collected to further analyze the effects of celecoxib on dystrophic muscle fibers. We first determined the potential for celecoxib to improve the sarcolemmal integrity of mdx muscle fibers. To this end, we immunostained TA muscle cross-sections with IgM, an extracellular protein that originates from the blood stream. If the integrity of the sarcolemma is compromised as in mdx mice, IgM penetrates within muscle fibers (Miura et al., 2009; Péladeau et al., 2016; Straub et al., 1997). Accordingly, IgM staining represents an excellent marker of sarcolemmal integrity. As illustrated in Figure 19, muscle fibers from wildtype mice showed little to no intracellular IgM staining. By contrast, fibers from mdx mice display a much larger proportion of fibers positively stained for IgM. In agreement with our grip strength data, IgM infiltration within dystrophic fibers was significantly reduced (~2-fold; P < 0.01) in TA muscle fibers following celecoxib treatment as compared to vehicle-treated mdx mice.
Figure 18. The effects of celecoxib treatment on muscle strength of mdx mice. A) mdx and wildtype mouse body weight (g) measured at different days from pre-injection (p-i) of celecoxib until the last day of treatment B) Analysis of forelimb grip strength of wildtype mice, vehicletreated or celecoxib-treated mdx mice, either normalized (right) or not (left) to body weight. N=6. Error bars represent SEM. *P < 0.05 and **P < 0.01 versus respective control.
A

Average mouse weight (g)

WT
mdx + veh
mdx + Cel

Time (days)

B

Forelimb grip strength (gF)

WT
mdx + veh
mdx + Cel

Forelimb Grip Strength / weight (g)

WT
mdx + veh
mdx + Cel
Figure 19. The impact of celecoxib treatment on the integrity of dystrophic muscle fibers. A) Cross-sections of TA muscle from wildtype mice and mdx mice treated with celecoxib or vehicle control, that were immunostained with goat-anti mouse IgM Alexa 594. B) Quantification of the amount of positive IgM fibers presented as a percentage to total TA muscle fibers. N=6. Error bars represent SEM, *P < 0.05, **P < 0.01 and ***P < 0.001 versus respective control. Scale bar = 50 μm.
Celecoxib treatment promotes expression of a slower, more oxidative phenotype in dystrophic muscles.

It is well established that mdx mice and DMD patients show decreased damage in their slow, oxidative muscle fibers compared to their fast, glycolytic counterparts (Gramolini et al., 2001a). Over the years, our laboratory and others have shown that promoting a switch to a slower phenotype provides key morphological and functional benefits to dystrophic muscle (Al-Rewashdy et al., 2015; Chalkiadaki et al., 2014; Ehrenborg and Krook, 2009; Gordon et al., 2013; Jørgensen et al., 2007; Ljubicic et al., 2011, 2014; Luquet et al., 2003; Miura et al., 2009; Reyes et al., 2015; Risérus et al., 2008; Wang et al., 2004; Winder et al., 2000). Therefore, to determine the expression profile of slow, oxidative versus fast, glycolytic markers in mdx mouse muscle treated with celecoxib or vehicle, we first conducted immunofluorescence experiments on cross-sections from TA muscles. Using antibodies against MHC isoforms, we found that a 4-week treatment with celecoxib caused a significant increase (P < 0.05) in the percentage of MHC type IIa fibers compared to vehicle-treated mdx mice (Figure 20A, B). In agreement with these data, western blotting revealed that expression of the OXPHOS markers, complex proteins IV and V, were significantly (P < 0.001) elevated in TA muscles from celecoxib-treated mdx mice (Figure 20C, D). Complex II protein levels in celecoxib-treated TA muscles also showed a trend towards an increase (P > 0.05) (Figure 20C, D). Together, these findings indicate that a 4-week treatment of celecoxib promotes expression of a slower, more oxidative myogenic program in dystrophic muscle.

DMD induces significant muscle damage that leads to severe inflammatory events and prominent immune cell infiltration between muscle cells (Nitahara-Kasahara et al., 2016). It has
Figure 20. The impact of celecoxib treatment on fiber type of TA mdx muscles. A) Representation of TA muscle cross-sections immunostained with MHC I, MHC Ila, MHC Ilb and embryonic MHC (eMHC) (red) in wildtype mice and mdx mice treated with celecoxib or vehicle control. Laminin is used to detect the muscle fiber sarcolemma (green) B) Graphical summary of the percentage of MHC type I, Ila, Ilb and eMHC positive fibers to total TA muscle fibers. C and D) Representative western blots and quantification of the expression of OXPHOS markers (complex II, complex IV, and complex V) in wildtype, celecoxib or vehicle treated mdx TA muscles. N=4-5. Scale bar = 50 μm. Error bars represent SEM. *P < 0.05 **P < 0.01 and ***P < 0.001 versus respective control.
been previously shown that the degree of inflammatory cell infiltration in dystrophic muscles is strongly associated with disease severity in patients (Nitahara-Kasahara et al., 2016). Since celecoxib has been shown to have anti-inflammatory properties (Brueggemann et al., 2010), we also determined whether the 4-week celecoxib treatment decreased immune cell infiltration in mdx mouse muscle. To this end, we immunostained TA muscle cross-sections from wildtype controls, celecoxib- and vehicle-treated mdx mice, using an antibody against CD11b, a commonly used immune cell marker (Fink et al., 2014; Holt et al., 2008). Compared to wildtype, mdx mouse muscles showed, as expected, significant immune cell infiltration (Figure 21). Celecoxib induced a marked reduction (~1.6-fold; P < 0.05) in CD11b-positive stained cells highlighting an overall decrease in immune cell infiltration in mdx mouse muscle following treatment (Figure 21A, B).

**Celecoxib treatment affects the morphology of mdx muscle fibers.**

It is well established that mdx mice have an abnormal assortment of small and large muscle fibers (Briguet et al., 2004; Torres and Duchen, 1987). Thus, we investigated the effects of celecoxib treatment on the morphology of dystrophic muscle fibers by performing haematoxylin and eosin (H and E) staining on TA cross-sections from wildtype as well as celecoxib- and vehicle-treated mdx mice. Analysis of the CSA of individual TA muscle fibers from celecoxib-treated mdx mice reveal increased frequency of small fibers (P < 0.05) and a clear trend towards an increase of particularly large fibers compared to vehicle-treated mdx animals. This results in an overall increase in the median CSA (Figure 22A, B). The increase in some fibers showing signs of hypertrophy might be surprising considering COX-2 inhibitors have been shown to reduce skeletal muscle hypertrophy in mice (Novak et al., 2009). However, such an increase in very large fibers was also seen in the TA and diaphragm muscles of mdx mice following a 4-week treatment with
Figure 21. The impact of celecoxib treatment on immune cell infiltration between dystrophic muscle fibers. A) TA muscle cross-sections immunostained with CD11b (red) in wildtype and mdx mice treated with celecoxib or vehicle control. Laminin stains the sarcolemma (green) B) Graphical summary of CD11b positive cells. N=4-5. Scale bar = 50 μm. Error bars represent SEM. *P < 0.05 and ***P < 0.001 versus respective control.
A

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B

![Graph showing CD11 positive cells for WT, mdx + veh, and mdx + Cel.](Graph_CD11.png)

* Note: Graph and images are placeholders for actual visual content.
the p38 MAPK activating drug heparin (Péladeau et al., 2016). To complement these data, we calculated the variance coefficient based on individual CSA of muscle fibers. Our results show that the variance coefficient of celecoxib treated TA fibers trends toward a decrease but did not reach significance (P > 0.05) (Figure 22C). We also measured central nucleation in TA muscles since this is a marker of muscle regeneration in dystrophic muscle fibers (Briguet et al., 2004). Our results show that celecoxib treatment did not modify the percentage of central nucleation compared to vehicle control (Figure 22D).

**Celecoxib increases levels of phosphorylated p38 and expression of utrophin A in cultured muscle cells.**

As previously mentioned, celecoxib is capable of activating p38 signaling in muscle (Hsiao et al., 2007; Steffel et al., 2006). Therefore, we verified that celecoxib indeed activates p38 MAPK in C2C12 differentiated myotubes, a convenient cell model to analyze the effects of celecoxib on myofibers in vitro. For this, we harvested myotubes treated with 5µM of celecoxib for 0-24 hours, and protein lysates were subjected to western blotting (Figure 23A). Quantification of the ratio between phospho-p38 and total-p38 protein levels shows that celecoxib induced a significant (P < 0.05) activation of p38 signaling in myotubes following 1, 4 and 8 hours of treatment (Figure 23B).

Based on our previous work showing that pharmacological activation of p38 can stimulate utrophin A expression levels (Amirouche et al., 2013; Péladeau et al., 2016), we also examined whether celecoxib increased utrophin A expression in cultured muscle cells. Myotubes were treated for 24 hours with 5 to 5000nM of celecoxib and lysates were processed for Western blots. Our results reveal that 24-hour celecoxib treatment of myotubes induced a dose-dependent increase in utrophin A levels, with a marked augmentation seen with a dose of 5000 nM (~2.5-fold; P < 0.05)
Figure 22. The effects of celecoxib treatment on the morphology of dystrophic muscles. A) Cross-sectional representations of TA muscles from wildtype and mdx mice treated with celecoxib or vehicle control, stained using hematoxylin and eosin. B) Graphical summary of CSA. Median CSAs of each muscle are displayed above the frequency histograms. Error bars represent SEM, *P < 0.05, significantly different from mdx vehicle control. N=6. (C)Variance coefficient measurements of the TA muscle fibers. (D) Percentage of central nucleation in TA muscle fibers. N=6. Error bars represent SEM **P < 0.01, ***P < 0.001 versus respective control. Scale bar = 50 μm.
Figure 23. The effects of celecoxib treatment on utrophin A and phosphorylated p38 expression levels in C2C12 myotubes. 

A) Representative western blot demonstrating activation of p38 MAPK upon celecoxib treatment (5000nM) in C2C12 myotubes, harvested at 0-24 hours. 

B) Quantification of phospho-p38 protein levels relative to total-p38 and normalized to β-actin. 

C) Representative western blot of utrophin A and α-tubulin protein levels using extracts from C2C12 myotubes treated with vehicle control or celecoxib (5, 50, 500 or 5000nM) for 24 h. 

D) Quantification of utrophin A protein levels normalized to α-tubulin as shown in (C). 

E) Relative quantification of utrophin A mRNA levels, normalized to 18S, as determined by qRT-PCR. N=3. Error bars represent SEM. *P < 0.05, **P < 0.01 versus vehicle control.
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B

Ratio of phospho p38/p38 total protein levels

Time (h)

0 1 4 8 24

Vehicle Celecoxib

C

Celecoxib

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Urotrophin

α-tubulin

D

Urotrophin A protein levels

veh 50 nM 500 nM 5000 nM

Celecoxib

E

Urotrophin A mRNA levels in myotubes (normalized to 18S)

veh Celecoxib 5000 nM
(Figure 23C, D). However, this celecoxib treatment failed to induce a parallel change in the expression of utrophin A mRNAs (Figure 23E).

Celecoxib treatment increases utrophin A protein levels in dystrophic skeletal muscles.

To determine if utrophin A levels were affected by celecoxib treatment in vivo, we performed western blotting with TA, diaphragm and heart muscles from wildtype mice, and vehicle- versus celecoxib-treated mdx mice. Our results revealed that muscles from mdx mice treated with celecoxib for 4 weeks showed an increase (~1.5 to 2-fold; P < 0.01 and P < 0.05) in utrophin A protein levels in TA, diaphragm and heart muscles compared to vehicle-treated mdx mice (Figure 24A, B). As observed with cultured C2C12 myotubes, this celecoxib treatment did not elicit (P > 0.05) an increase in utrophin A mRNA levels in the TA (Figure 24C), in the heart [(Utrophin A mRNA levels normalized to 18S: WT = 1.00, mdx + vehicle = 1.05, mdx + celecoxib = 1.2, ±0.37) (mean and SEM)] or diaphragm [(Utrophin A mRNA levels normalized to 18S: WT = 1.00, mdx + vehicle = 1.0, mdx + celecoxib = 1.15, (Ut147) (mean and SEM)].
Figure 24. Utrophin A expression levels in skeletal muscle in response to celecoxib treatment.

A and B) Representative western blots and quantification of utrophin A and β-actin protein levels in the tibialis anterior (TA), the diaphragm (DIA) and the heart of 6-week-old wildtype and mdx mice treated with celecoxib (20µg/kg) or vehicle for 4 weeks. C) Relative quantification of utrophin A mRNA levels, normalized to 18S, in the TA muscles as determined by qRT-PCR. N=4-5. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001, versus respective control.
Discussion

In the present study, we explored the therapeutic potential of the FDA-approved drug celecoxib for the treatment of DMD. Since a variety of NSAIDs have been recognized to improve muscle function and morphology (Miyatake et al., 2016; Serra et al., 2012), we started by analyzing the ability of celecoxib to ameliorate the dystrophic muscle phenotype. Thus, we performed a 4-week treatment with celecoxib in 6-week-old mdx mice. First, we found that this treatment attenuated the sarcolemmal damage of mdx muscle fibers, which constitutes one of the earliest and striking characteristics of dystrophin deficiency in DMD patients and mdx mice (Dudley et al., 2006; Mokri and Engel, 1975). This resulted in overall improvement of mdx mouse muscle function and strength. Second, the celecoxib treatment significantly decreased immune cell infiltration between muscle fibers, most likely as a result of its anti-inflammatory properties by preventing the generation of prostaglandins (Daham et al., 2011; Davies et al., 2000; Dubois et al., 1998; Vane, 1971). Third, we demonstrate that the beneficial effects of celecoxib on the progression of the disease may be in part due to its ability to alter muscle fiber morphology and promotion of a slower, more oxidative phenotype in muscle. In this context, it is well established that mdx mice and DMD patients show decreased damage in their slow, oxidative muscle fibers compared to their fast, glycolytic muscle fibers (Gramolini et al., 2001a). Our laboratory and others have shown that promoting a switch to a slower phenotype provides key morphological and functional benefits to dystrophic muscle (Al-Rewashdy et al., 2015; Chalkiadaki et al., 2014; Ehrenborg and Krook, 2009; Gordon et al., 2013; Jørgensen et al., 2007; Ljubicic et al., 2011, 2014; Luquet et al., 2003; Miura et al., 2009; Reyes et al., 2015; Risérus et al., 2008; Wang et al., 2004; Winder et al., 2000). Finally, these beneficial adaptations to celecoxib treatment were
accompanied by an upregulation of utrophin A. Together, these findings indicate that celecoxib may thus represent a novel and attractive therapeutic for treating DMD.

**Celecoxib treatment stimulates utrophin A expression in skeletal muscle.**

An upregulation of utrophin A protein in mdx mouse muscle has been shown to improve their function and integrity by binding and stabilizing the DAPC at the sarcolemma (Ishikawa-Sakurai et al., 2004a; Janghra et al., 2016; Peters et al., 1998a). Furthermore, our laboratory was the first to demonstrate that slow, oxidative myofibers express significantly more utrophin A compared to fast glycolytic fibers (Gramolini et al., 2001a). Our current results show that celecoxib treatment induced a ~2.5-fold (P < 0.05) increase in utrophin A protein levels in cultured skeletal muscle cells and a ~1.5 to 2-fold (P < 0.05) increase in TA, diaphragm and heart muscles from mdx mice. These data are important since it is now well recognized that a 2-fold upregulation of utrophin A in muscle fibers is sufficient to improve the dystrophic phenotype (Tinsley et al., 1998). Moreover, this increase in utrophin A protein expression following celecoxib treatment fits nicely with the switch to a slower, more oxidative fiber type in mdx mice, thereby further highlighting the close link between utrophin A levels and muscle phenotypes.

Based on these results, we sought to also decipher possible mechanisms by which celecoxib increases utrophin A protein levels. We recently demonstrated that utrophin A protein levels can be altered by a post-transcriptional event involving KH-type splicing regulatory protein (KSRP) binding to the 3’UTR of utrophin A transcripts, promoting their degradation (Amirouche et al., 2013). In this earlier work, we also showed that pharmacological activation of p38 MAPK by heparin decreased the functional availability of KSRP to bind to the 3’UTR of utrophin A mRNAs, thereby increasing their stability and inducing greater protein expression (Amirouche et al., 2013;
Péladeau et al., 2016). However, our current results demonstrate that even though celecoxib treatment did cause an activation of p38 MAPK signaling in muscle cells as previously shown by others (Farooq et al., 2013; Hsiao et al., 2007; Steffel et al., 2006), as well as an increase in utrophin A protein expression, celecoxib failed to alter utrophin A mRNA levels. In parallel, we also observed that celecoxib treatment did not affect KS RP protein levels, contrary to what we had previously seen following heparin treatment, nor did it affect expression of other AU-rich binding-proteins including TTP and AUF1 (Figure 25A). Taken together, these findings suggest that an alternate pathway to that of heparin treatment is involved in the celecoxib-induced stimulation of utrophin A protein expression.

In light of the absence of an increase in utrophin A mRNA levels following celecoxib treatment, two possible mechanisms might be envisaged; either a translational mechanism or modifications in protein stability. In this context, recent studies from our lab revealed that utrophin A protein expression can be regulated through an IRES-mediated translational event (Miura et al., 2005, 2008, 2010). Importantly, this finding was recently confirmed by others (Ghosh and Basu, 2015). Together, these studies showed that indeed, utrophin A transcripts contain an IRES in their 5’UTR that can drive cap-independent translation in skeletal muscle cells (Miura et al., 2010). It thus appears likely that under our conditions, celecoxib treatment activates cap-independent translation of utrophin A through the regulation of key signaling proteins. One possible candidate in such a scenario is the RNA-binding protein HuR. In fact, HuR was previously reported to promote translation of target mRNAs by interacting with IRES sequences located in the 5’UTR (Durie et al., 2011; Srikantan et al., 2012). Moreover, HuR can also be activated by p38 MAPK to stabilize target mRNAs (Farooq et al., 2009, 2013; Lafarga et al., 2009; Slone et al., 2016) and in this context, Farooq et al. showed that HuR is required for the SMN protein increase seen in
celecoxib-treated NT2 cells (Farooq et al., 2013). In our hands, we observed that celecoxib induced a marked ~2 and 4-fold increase (P < 0.05) in HuR protein levels in both TA and diaphragm muscles, respectively (Figure 25B). Furthermore, a RNA-immunoprecipitation assay demonstrates that HuR is able to bind to utrophin A mRNAs in skeletal muscle cells (P < 0.001) (Figure 25C). Collectively, these data suggest that celecoxib stimulates HuR protein expression in muscle, which in turn may facilitate interaction of HuR with the utrophin A IRES culminating in increased translation of utrophin A. Although such a series of events appears reasonable, additional work is nonetheless necessary to better define the apparent link between p38 activation, HuR and translation of utrophin A.

**Repurposing celecoxib for the treatment of DMD.**

Glucocorticoids have been shown to have beneficial effects on dystrophic muscles and are currently the only therapy for DMD patients. Studies from our lab and others have shown that glucocorticoid treatment results in the stimulation of utrophin A expression in skeletal muscle fibers (Courdier-Fruh et al., 2002; Pasquini et al., 1995; St-Pierre et al., 2004) through, as discussed above, translational events (Miura et al., 2008). Despite positive outcomes, these drugs are associated with significant side effects (Moxley et al., 2010). It is thus important to find additional drugs such as NSAIDs that can improve the dystrophic phenotype in DMD patients without having the harmful side effects of steroids. In the present study, we have demonstrated the many positive effects of the NSAID celecoxib, used at a low dose of 20µg/kg. It did not come as a surprise that a low dose could have a significant effect in mdx mouse muscles, since treatment with a low celecoxib dose has been proven to be sufficient to have beneficial effects in SMA mice by activating SMN expression, and in cancer cells by regulating the cell cycle and apoptosis.
Figure 25. Common RNA-binding protein expression levels in skeletal muscle in response to celecoxib treatment. Representative western blots and quantification of A) KSRP, TTP, AUF1, B) HuR and β-actin protein levels in the TA and the diaphragm (DIA) from wildtype and mdx mice treated with celecoxib or vehicle control for 4 weeks. N=4-5. C) C2C12 cells lysates were subjected to an RNA immunoprecipitation experiment using a HuR antibody. Immunoprecipitation was verified by western blot with the same HuR antibody. Co-immunoprecipitated utrophin A mRNAs were detected by qRT-PCR with GAPDH mRNAs as a house keeping gene. N=4. Error bars represent SEM, *P < 0.05, ***P < 0.001, significantly different from respective control.
It is important to note that at higher doses, celecoxib treatment can have damaging side effects such as increased likelihood of cardiovascular thrombotic events, as well as liver and gastrointestinal lesions (Davies et al., 2000; Drmic et al., 2017; Mukthinuthalapati et al., 2017). However, when converting the dose required for the attenuation of the dystrophic pathology in mice to an equivalent human dose by adjusting for differences in body surface area (Nair and Jacob, 2016), we obtain a dose approximately 100 times smaller than the recommended oral dose for patients (1-5mg/kg/twice daily). Thus, it appears likely that damaging side effects of celecoxib would be limited in DMD (Davies et al., 2000; Simon et al., 1998). Furthermore, over the years the pharmacokinetics and pharmacodynamics of celecoxib have been carefully studied, and were shown to elicit only minor differences between species (Gong et al., 2012; Paulson et al., 1999, 2000). Thus, we are optimistic that the effects of celecoxib in mice could be replicated in humans.

Since both glucocorticoids and celecoxib can activate utrophin A and muscle function in mdx mice, an interesting therapeutic approach could also be a combinatorial treatment. Along those lines, we have recently demonstrated that treating mdx mice with two potent utrophin A activating drugs that stimulate distinct pathways can have an additive effect on utrophin A levels (Péladeau et al., 2016). More interestingly, it has been shown that inhibition of COX-2 by celecoxib enhances glucocorticoid receptor function in neuronal PC12 cells (Hu et al., 2005). Thus, a combinatorial treatment with glucocorticoids and celecoxib could indeed have either additive or even synergistic effects for DMD patients.

Repurposing an FDA-approved drug such as celecoxib will accelerate the development and implementation of novel therapeutics for treating DMD patients. In fact, over the last several years, we have focused our work on repurposing FDA-approved or clinically tested drugs to determine
their beneficial effects on dystrophic mice and utrophin A upregulation in skeletal muscles (Amirouche et al., 2013; Ljubicic and Jasmin, 2015; Ljubicic et al., 2011, 2014; Miura et al., 2009; Péladeau et al., 2016). This pharmacological strategy permits easier administration and systemic delivery to all DMD patients regardless of their dystrophin mutations. Additionally, such an approach limits toxicological concerns due to previously established doses for use of these drugs and their known side effects. Our current findings show that a low dose celecoxib treatment improves function and promotes expression of utrophin A in mdx mouse muscle, and indicate that celecoxib may represent a promising therapeutic for the treatment of DMD, which can also be linked to beneficial anti-inflammatory effects.
Materials and Methods

Celecoxib Treatment.

Mouse C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were cultured according to manufacturer instructions and as described previously (Péladeau et al., 2016). Cells were seeded in 6-well matrigel-coated culture dishes and differentiated for 96 hours in 2% horse serum in DMEM. Myotubes were then treated with celecoxib (Celebrex) (Cayman Chemical, Michigan, USA) (5nM, 50nM, 500nM, 5000nM) or DMSO (control) for 24 hours as used in an earlier study (Amirouche et al., 2013; Irrcher et al., 2008).

In vivo experiments were performed using C57BL/10ScSn-Dmdmdx/J (mdx) mice and C57BL/10 (wild-type) mice (The Jackson Laboratory, Bar Harbor, USA), which were maintained under a constant 12-hour light-dark cycle with continuous access to water and food. Six week-old mdx mice were treated daily with celecoxib (20μg/kg), a dose recently used by others (Farooq et al., 2013), or vehicle control (saline) by intraperitoneal injections, for a 4-week period (Amirouche et al., 2013; Jørgensen et al., 2007; Ljubicic et al., 2011; Zbinden-Foncea et al., 2012). Muscles were dissected from euthanized mice, flash frozen in liquid nitrogen or melting isopentane for further analysis.

Forelimb grip strength.

On the final day of treatment, forelimb grip strength performance of the mice was assessed using a digital force gauge, Chatillon DFE II (Columbus Instruments, Columbus, USA), with a grid. Before the test began, the mice were acclimatized to the work area for 30 minutes. Each mouse was allowed to grip the grid firmly and was pulled horizontally away from the bar at a...
speed of 2.5cm/second, until it released the grid. The value of the maximal peak force was recorded (gF). This procedure was repeated six times per mouse, with a 30 second rest time between measurements. All grip strength measurements were conducted by the same investigator to limit variability. These experiments were also blinded by using a second investigator who was handing out cages to the one doing the testing.

**Western Blotting.**

Proteins were extracted from C2C12 cells or powdered muscles (homogenized with a BioPulverizer) in urea extraction buffer. Extraction and protein quantification procedures were performed as previously described (Péladeau et al., 2016). Five-fifteen µg of extracted proteins were loaded and separated on a 7% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada). Membranes were then stained with Ponceau to confirm equal loading and blocked as described previously (Péladeau et al., 2016). Blots were incubated with antibodies directed against utrophin A (1:500; Novocastra, Leica biosystems, Concord, ON, Canada), oxidative phosphorylation markers (OXPHOS) cocktail (1:1000; abcam, Toronto, ON, Canada), phosphorylated or total p38 MAPK (1:1000 Cell Signaling, Danvers, MA, USA), KSRP (1:1000; Bethyl Laboratories, Montgomery, TX, USA), Tristetraprolin (TTP) (1:1000; Santa Cruz, Dallas, Texas) AU-binding factor 1 (AUF1) (1:1000; EMD Millipore, Ontario, Canada) or HuR (1:1000; Santa Cruz, Dallas, Texas) and incubated with the appropriate Horse Radish Peroxidase-conjugated secondary antibody. Either β-actin or α-tubulin antibodies (1:10000; Santa Cruz, Dallas, Texas, USA) were used as a loading controls. Chemiluminescent detection of proteins was performed using ECL reagent (Perkin Elmer,
Waltham, MA, USA). The films were quantified using ImageJ (NIH version 1.0) and/or Image Lab.

**Immunofluorescence, Haematoxylin and Eosin Staining.**

Ten μm TA muscle cross-sections were prepared for immunofluorescence using a Mouse on Mouse immunodetection kit (Vector Laboratories, Burlington, ON, Canada). For fiber typing analysis, sections were incubated with primary antibodies against myosin heavy chain (MHC) isoforms including MHC I (BA-F8), IIa (SC-71), IIB (BF-F3) and embryonic (eMHC) (F1.652) (Hybridoma Bank, Iowa city, IA, USA). A Texas Red conjugated streptavidin antibody (1:500; Vector laboratories, Burlington, ON, Canada) was used for detection of the MHC isoforms. To analyze immune cell infiltration between muscle fibers, cross-sections were incubated with a CD11b antibody (1:100; BD Biosciences, California, USA) and incubated with its appropriate secondary antibody as described above. Immunoglobulin M (IgM) staining was performed using a FITC-conjugated IgM anti-mouse secondary antibody (1:400; Sigma-Aldrich, Oakville, Canada). All sections were co-stained with a rabbit laminin antibody (1:800; Sigma-Aldrich, Oakville, Canada) and a goat anti-rabbit Alexa Fluor 488 IgG secondary antibody (1:500; ThermoFisher Scientific, Massachusetts, USA) to visualize the muscle fiber sarcolemma. The slides were mounted with Vectashield containing DAPI staining (Vector Laboratories, Burlington, ON, Canada) and visualized using a Zeiss Axioskop-2 microscope. Quantification was accomplished using Image J (NIH version 1.0).

TA muscle cross-sections were stained with Hematoxylin and Eosin dyes followed by dehydration using ethanol wash solutions. The sections were cleared with toluene and mounted using Permount (Fisher Scientific, Ottawa, Canada). The slides were visualized as described
above. Percentage of central nucleation was determined by counting the total number of muscle fibers and the number of muscle fibers with centrally-located nuclei from 6 cross-sectional views using the Northern Eclipse Software (NES, EMPIX Imaging, Mississauga, Ontario, Canada). Cross-sectional Area (CSA) of each muscle fiber was also measured from 6 cross-sectional views using the NES Software. The variance coefficient was calculated based on the CSA of muscle fibers using the formula “variance coefficient Z = 1000 x standard deviation of muscle fiber CSA/mean muscle fiber CSA”.

**RNA Extraction and qRT-PCR.**

RNA extraction, reverse transcription and qRT-PCR were performed as previously described (Péladeau et al., 2016). Amplification of utrophin A and the 18S ribosomal subunit was performed with the following primer sequences: utrophin A - forward 5′-ATCTTGTCGGGCTTTCCAC-3′ and reverse 5′-ATCCAAAGGGCTTTCCCAGAT-3′, 18S ribosomal subunit- forward 5′-CGCCGCTAGAGGTGAAATC-3′ and reverse 5′-CCAGTCGGCATCGTTTATGG-3′

**RNA-immunoprecipitation.**

Skeletal muscle C2C12 cells were seeded on 150 mm culture plates and incubated at 37 °C with 5% CO₂ in a humidified chamber, for 24 hours. The cells were washed with 1X PBS pH 7.4, fixed in 1% formaldehyde for 10 minutes, and the reaction was quenched with a 0.25 M glycine solution in PBS pH 7.4 for 5 minutes as described previously (Parks et al., 2017). Cells were washed with PBS, resuspended in RIPA Buffer, sonicated for 4 × 15 seconds and centrifuged (16000 xg) for 10 minutes at 4 °C. Lysates were pre-cleared with protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and a blocking solution (0.08 µg/µl of salmon sperm DNA and 0.4 µg/µl of competitor tRNA). Beads were removed by centrifugation and the pre-cleared lysate was aliquoted (a small input aliquot and the rest for an IgG and IP aliquot).
HuR antibody (Santa Cruz, Dallas, Texas) (3µg) or IgG control and protein A/G plus agarose beads incubated with gentle rotation at 4 °C for 1 hour. Antibody-bound beads and the pre-cleared lysates were then left to immunoprecipitate overnight at 4 °C with gentle rotation. Beads were washed with RIPA buffer (5 times for 10 minutes) and then washed with TE Buffer (10mM Tric-HCl pH7.4, 1mM EDTA) (2 times for 5 minutes). The collected beads were resuspended in 100 µL of Elution Buffer (50mM Tris-HCl pH7.4, 10mM DTT, 5mM EDTA, 1% SDS). Following the resuspension, crosslinking was reversed (5 hours at 70°C). Immunoprecipitation efficiency was determined by Western blotting and RNA levels were determine by qRT-PCR.

Statistical Analysis.

The data were analyzed using unpaired student’s t-test and one-way ANOVAs (Analysis of Variance) with Bonferroni post-hoc tests. Error bars represent standard error of the mean (SEM). Statistical analysis was done with Graph Pad prism 5 (Prism Software, La Jolla, CA, USA). Significance was accepted at *P ≤ 0.05.
Acknowledgements

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Chapter 4
Identification of novel therapeutics that target eEF1A2 and upregulate utrophin A translation in dystrophic muscles

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Author’s contribution:

Conceived and designed the experiments: CP, BJJ. Performed the experiments: CP, NA, AC, HA
Analyzed the data: CP, NA. Provided expertise: LMB, AM, MH. Contributed reagents/materials/analysis tools: JV, AM, MH. Wrote the Manuscript: CP, BJJ. Contributions by figure: Fig 26 (AC, LMB), Fig 27 (CP, HA), Fig 28 (CP), Fig 29 (CP, NA), Fig 30 (CP, NA), Fig 31 (CP, NA), Fig 32 (CP, NA), Fig 33 (CP)
Abstract

Up-regulation of the dystrophin homologue utrophin in skeletal muscle represents a promising therapeutic strategy for the treatment of Duchenne Muscular Dystrophy (DMD). Thus, understanding the underlying regulatory mechanisms that control utrophin A expression is important. We previously demonstrated that the eukaryotic elongation factor 1A2 (eEF1A2) associates with the 5’UTR of utrophin A to promote internal ribosome entry site (IRES)-dependent translation. Here, we conducted a comprehensive series of experiments to: 1) examine whether eEF1A2 directly regulates utrophin A expression in mouse muscle in vivo; 2) identify via an in-cell ELISA-based high-throughput screen FDA-approved drugs that upregulate both eEF1A2 and utrophin A; and 3) characterize therapeutic potential of leads in mdx mice. To this end, we first used multiple mouse models to determine the physiological role of eEF1A2 in the regulation of utrophin A in skeletal muscle and show that direct transient overexpression of eEF1A2 in wild-type and mdx skeletal muscles causes a marked increase in endogenous utrophin A protein. In addition, overexpression of eEF1A2 in muscles of transgenic mice harboring a bicistronic construct containing the utrophin A 5’UTR led to activation of IRES-mediated translation. Based on these findings, we designed an in vitro high-throughput drug screen and discovered seven classes of FDA-approved drugs that increase both eEF1A2 and utrophin A protein levels in skeletal muscle cells. Treatment of mdx mice with the 2 top leads, Betaxolol and Pravastatin, showed improvements in muscle strength, increases in utrophin A protein expression at the sarcolemma and a marked amelioration in the integrity of muscle fibers. Finally, we show that drug-induced utrophin A upregulation is attenuated in eEF1A2-null mice (wasted mice), highlighting the key role of eEF1A2 in mediating the induction of utrophin A. Collectively, our findings show that IRES-mediated translation of utrophin A via eEF1A2 is a critical mechanism of regulating...
Introducing utrophin A expression and reveal the potential of repurposed drugs for treating DMD via this pathway.

**Introduction**

Duchenne muscular dystrophy (DMD) is the most common hereditary debilitating muscle disease, and is caused by the absence of dystrophin protein in skeletal muscle (Blake et al., 2002; Emery, 1991). The major functional role of dystrophin is to create a link between the internal cytoskeletal actin network and the extracellular matrix in order to provide structural integrity to the sarcolemma of muscle fibers (Ervasti, 2007; Ervasti et al., 1990; Hoffman et al., 1987; Koenig and Kunkel, 1990). Skeletal muscle fibers lacking dystrophin, as observed in DMD patients, display a higher susceptibility to stress-induced sarcolemmal injury, extracellular calcium influx in muscle, increased inflammation and replacement of muscle fibers by connective and adipose tissues (Birnkrant et al., 2018a, 2018b, 2018c; Miyatake et al., 2016; Schaecher et al., 2004). DMD patients eventually succumb to the disease by early adulthood due to cardiac or respiratory failure (Bach et al., 1987; Guiraud et al., 2015a). Despite the fact that over 200 promising studies, diagnostic tests and treatment trials are currently in various stages of enrollment (the NIH Clinical Trials.gov), there is still no effective treatment. One potential therapy aims to increase the sarcolemmal expression of utrophin A, the autosomal homologue of dystrophin, that can functionally compensate for its loss in muscles of several animal models of DMD (Cerletti et al., 2003; Fisher et al., 2001; Kennedy et al., 2018; Tinsley et al., 1998, 1996). Several studies have indeed demonstrated using either transgenic (Cerletti et al., 2003; Fisher et al., 2001; Kennedy et al., 2018; Rafael et al., 1998; Tinsley et al., 1998, 1996) or pharmacological (Al-Rewashdy et al., 2015; Consalvi et al., 2013; Guiraud and Davies, 2017; Ljubicic et al., 2011, 2014; Miura et al.,...
strategies that enhancing expression of utrophin A can alleviate numerous pathophysiological features of DMD and can thus be of great therapeutic benefit.

Over the years, multiple studies have focused on determining the key transcriptional regulatory mechanisms that control utrophin A expression in muscle (Angus et al., 2005; Chakkalakal et al., 2003; Dennis et al., 1996; Galvagni et al., 2001, 2002; Gramolini and Jasmin, 1999; Gramolini et al., 1997, 1999; Gyrd-Hansen et al., 2002; Khurana et al., 1999; Perkins et al., 2001; Stocksley et al., 2005). However, recent evidence demonstrates the importance of post-transcriptional and translational events in the regulation of utrophin A. In fact, expression of utrophin A has been shown to be highly regulated at its 3’ end, where cis-elements promote the stability of utrophin A mRNA transcripts (Amirouche et al., 2013; Bonet-Kerrache et al., 2005). In contrast, other studies have demonstrated discordance between utrophin A protein and mRNA levels in DMD muscle biopsy samples and mouse regenerating muscle fibers, suggesting that utrophin A expression is also regulated at the translational level (Amenta et al., 2011; Basu et al., 2011; Gramolini et al., 1999; Weir et al., 2002). In this context, the utrophin gene can produce two full-length isoforms, utrophin A and utrophin B, which are transcribed from distinct promoters and have different 5’-untranslated regions (5’-UTRs) (Burton et al., 1999; Dennis et al., 1996). Both proteins are identical, except for N-terminal regions (Burton et al., 1999). The 5’UTR of utrophin A, the skeletal muscle isoform, is long and CG-rich which suggests that utrophin A can indeed be subjected to translational control as long CG-rich elements can reduce efficiency of conventional scanning from the 5’-end during cap-dependent protein translation (Dennis et al., 1996; Ghosh and Basu, 2015; Komar and Hatzoglou, 2011; Miura et al., 2005). Our laboratory has discovered some years ago the presence of an internal ribosome entry site (IRES) within the 5’UTR of utrophin A that promotes expression through IRES-dependent translational mechanisms (Miura et al., 2005,
2010). Of relevance, our initial findings have been confirmed by others (Ghosh and Basu, 2015) and, more recently, an IRES was also found in dystrophin (Wein et al., 2014).

The rate-limiting step of cap-dependent translational initiation is the binding of the eukaryotic initiation factor (EIF) 4F protein complex to the 7-methylguanylate cap (m\(^7\)G), also known as the 5’cap. Under certain cellular and physiological conditions, including disease or stress, IRES-dependent translation of mRNAs is enhanced while cap-dependent translation is simultaneously attenuated (King et al., 2010; Liwak et al., 2012). IRES elements are thought to associate with the translational machinery, including the canonical initiation factors, as well as IRES trans-acting factors (ITAFs), which enable the recruitment of the ribosome to initiate peptide synthesis (Komar and Hatzoglou, 2011; Spriggs et al., 2005). It has been suggested that ITAFs act as RNA chaperones to modulate IRES activity in the appropriate conformational formation to promote ribosome binding (Pickering et al., 2004). However, the precise mechanisms involved in IRES-dependent translation remain largely unknown.

Our laboratory previously demonstrated that muscles expressing a bicistronic reporter construct containing the utrophin A 5’UTR and subjected to degeneration and regeneration cycles by cardiotoxin injections, generated strong utrophin A IRES activity (Miura et al., 2005). In addition to potential translational events regulating utrophin A in regenerating fibers, our laboratory also demonstrated activation of this IRES following glucocorticoid treatment (Miura et al., 2005, 2008). Interestingly, this IRES appears capable of preferentially driving translation of utrophin A in skeletal muscle (Miura et al., 2010). Through a series of experiments including RNA-affinity chromatography, mass spectrometry and UV-crosslinking studies, we previously identified eEF1A2 as a putative ITAF able to modulate the activity of the utrophin A IRES (Miura et al., 2010). Our aims in the present study were three-fold. First, we wished to examine the role
of eEF1A2 in directly regulating the endogenous expression of utrophin A in muscle of several mouse models. Next, by performing a high-throughput drug screen, we sought to identify FDA-approved drugs that target eEF1A2, thereby upregulating utrophin A expression through IRES activation. Finally, we wanted to characterize the therapeutic potential of activating translation of utrophin A through eEF1A2 in mdx mouse muscle with leads identified in the screen. Collectively, our findings provide several complementary physiological lines of evidence indicating that targeting the activity of the utrophin A IRES is a viable strategy with potential therapeutic benefits for increasing endogenous expression of utrophin A in DMD muscle fibers. In addition, we identified several FDA-approved drugs that stimulate IRES-dependent translation of utrophin A through eEF1A2, with potential to accelerate the clinical implementation of novel therapeutics to treat DMD.
Results

Expression of eEF1A2 in fast and slow muscles of wild-type and mdx mice

In a first set of experiments, we examined whether the endogenous expression of eEF1A2 differs in wild-type versus mdx mice in fast EDL and slow soleus muscles. Mdx and wild-type mouse muscle lysates were used for western blot analyses. Results did not reveal any significant (P > 0.05) difference in the relative abundance of eEF1A2 protein content in fast and slow muscles of wild-type versus mdx mice (Figure 26A). This indicates that strategies aimed at further increasing the expression and/or activity of eEF1A2 in muscle may be of therapeutic benefit for DMD patients.

Overexpression of eEF1A2 in muscle increases expression of utrophin A and IRES activity

To determine the impact of eEF1A2 on utrophin A expression in vivo, we overexpressed eEF1A2 in skeletal muscle and analyzed utrophin A protein levels in both wild-type and mdx mice. To this end, we electroporated an eEF1A2-expressing construct (MYC-HIS360-eEF1A2-pcDNA) or pcDNA3.1 control into the tibialis anterior (TA) muscles of wild-type and mdx mice and harvested the muscles 7 days later. Western blot analyses showed a nearly 2-Fold increase (P < 0.05) in utrophin A protein levels in wild-type and mdx mouse muscles overexpressing eEF1A2 as compared to controls (Figure 26B).

In these experiments, it was important to determine whether the increase in utrophin A protein levels seen following eEF1A2 overexpression is mediated via activation of the utrophin A IRES. Accordingly, we electroporated the eEF1A2-expressing construct into TA muscles of our transgenic mice previously generated in our lab (Miura et al., 2010), which harbor the CMV/β-GAL/UtrA/CAT bicistronic reporter transgene that contains the utrophin A 5’UTR. In this
construct, the first cistron of β-GAL reflects cap-dependent translation whereas the second cistron (CAT) represents IRES-dependent translation regulated by the inserted utrophin A 5’UTR. Therefore, an increase in CAT activity accompanied by no changes in β-GAL, corresponds to an activation of utrophin A IRES-dependent translation. Following electroporation of the eEF1A2 construct into TA muscles, we performed a series of standard analyses to determine utrophin A IRES activity as shown by a ratio of CAT to β-GAL activity. Our analysis demonstrated a clear trend towards increased IRES reporter activity in muscles of our transgenic mice overexpressing eEF1A2 (Figure 26C). Due to the variability inherent to these experiments however, this change did not reach statistical significance (P = 0.19). However, examination of the averaged raw values of reporter activity in muscles overexpressing eEF1A2 and control showed, as expected for IRES activation, an increase in CAT (control; 161.3 ± 31.2 versus eEF1A2; 302.3 ± 33.3, P = 0.17), but little change in β-GAL (control 0.55; ± 0.12 versus eEF1A2; 0.61 ± 0.14, P = 0.76) activity. Collectively, these findings indicate that eEF1A2 directly increases endogenous utrophin A protein expression by acting via the utrophin A IRES.
Figure 26 - Overexpression of eEF1A2 in skeletal muscles increases endogenous utrophin A protein levels and utrophin A IRES activity. A) Representative western blot comparing the expression profile of eEF1A2 protein in fast (EDL) and slow (soleus) skeletal muscles harvested from 6-7-week-old wild-type (WT) and mdx mice. β-actin was used as a loading control (n = 3).

B) eEF1A2 (eEF1A2-pcDNA) expression construct or control (pcDNA3.1) were electroporated into TA muscles of 4-6-week-old wild-type and mdx mice as well as in TA muscles from utrophin A 5’UTR (CMV/βGAL/UtrA/CAT) reporter transgenic mice. One week later, muscles were harvested and prepared for western blot or reporter assays. Representative western blots of endogenous utrophin A protein expression in wild-type and mdx mice following overexpression of eEF1A2 with the respective quantification. Ponceau staining was used as a loading control. Note the increase in utrophin A protein levels in skeletal muscles overexpressing eEF1A2 (N = 3-6).

C) Relative IRES activity as determined by a ratio of CAT: β-GAL activity in TA muscles of utrophin A 5’UTR (CMV/βGAL/UtrA/CAT) transgenic mice overexpressing eEF1A2 (N = 9). Error bars represent SEM. *P < 0.05, versus control.
**Pharmacological activation of utrophin A expression through eEF1A2**

Based on our data demonstrating that overexpression of eEF1A2 stimulates utrophin A protein expression in skeletal muscle of wild-type and mdx mice, we sought to identify FDA-approved drugs that target eEF1A2 to activate utrophin A translation as a therapeutic approach for treating DMD. Accordingly, we designed an ELISA-based high-throughput drug screen with a total of 262 FDA-approved drugs. C2C12 myoblasts were treated with each drug or vehicle control for 24 hours. It is important to note that the drug doses used in this screen are clinically relevant. Following treatment, protein levels of both eEF1A2 and utrophin A were assessed. A drug was considered a hit if it had the ability to significantly raise both eEF1A2 and utrophin A protein levels over vehicle control. From this screen, we obtained 11 drugs that were considered hits (Table 3 and Figure 27A).

We noted that some of these FDA-approved drugs have common roles such as anti-diabetic, anti-peptic ulcer, cholesterol-lowering and beta-adrenergic blocking agents (Table 3). Interestingly, we found in this high-throughput screen that four drugs, Acarbose, Labetalol, Pravastatin, and Telbivudine (Table 4 and Figure 27A) caused significant and reproducible increases of at least ~2-fold in the levels of eEF1A2 and utrophin A. This is important because it has been shown that a 2-fold increase of utrophin A in muscle is sufficient to improve the dystrophic pathology (Tinsley et al., 1998). Subsequent confirmation of the effects of the 11 drugs was performed by treating C2C12 myoblasts with each drug at three different doses, including the dose used for the screen, for a period of 24 hours. Western blot analysis showed that under these conditions, expression of both utrophin A and eEF1A2 increased significantly (P < 0.05) to an extent similar or higher to that observed in the high-throughput screen, thereby validating our findings obtained with the screen (Table 4 and Figure 28). Furthermore, we observed that most of the 11
Table 3. eEF1A2 and utrophin A activating FDA-approved drugs identified using an ELISA-based high-throughput screen.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Compound family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosiglitazone</td>
<td>Anti-diabetic agent</td>
</tr>
<tr>
<td>Acarbose</td>
<td></td>
</tr>
<tr>
<td>Nizatidine</td>
<td>Anti-peptic ulcer agent</td>
</tr>
<tr>
<td>Olsalazine·Na</td>
<td></td>
</tr>
<tr>
<td>Lovastatin</td>
<td>Cholesterol-lowering agent</td>
</tr>
<tr>
<td>Pravastatin·Na</td>
<td></td>
</tr>
<tr>
<td>Betaxolol·HCl</td>
<td>Beta-adrenergic blocking agent</td>
</tr>
<tr>
<td>Labetalol</td>
<td></td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>Diuretic agent</td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>Anti-hyperthyroidism agent</td>
</tr>
<tr>
<td>Telbivudine</td>
<td>Anti-hepatitis B virus agent</td>
</tr>
</tbody>
</table>
Figure 27. Pharmacological activation of eEF1A2 and utrophin A by FDA-approved drugs.

A) An ELISA-based high-throughput drug screen was performed by treating C2C12 cells with 262 different FDA-approved drugs or vehicle control for 24 hours. Quantification of eEF1A2 and utrophin A protein levels from the 11 FDA-approved drugs considered as hits. A drug is considered a hit based on its ability to increase eEF1A2 and utrophin A’s protein levels over vehicle control.

C) Activation of the 5’UTR utrophin IRES reporter construct by 24-hour treatment of FDA-approved drugs in C2C12 cells. The treated muscle cell samples were subjected to a reporter assay to determine CAT and β-gal activity representative of IRES activity. CAT activation was normalized to β-gal and each drug activation was normalized to vehicle control.

D) Relative quantification of utrophin A mRNA levels in C2C12 treated cells, determined by qRT-PCR. N=3. The values were normalized to 18S mRNA levels. Error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001, versus vehicle control.
drugs promoted a dose-dependent response of eEF1A2 and utrophin A protein levels. This indicates that our screen to identify drugs targeting eEF1A2 and utrophin A proved to be successful to identify FDA-approved drugs that could be beneficial for the treatment of DMD.

*eEF1A2-targeting drugs stimulate utrophin A expression in cultured myoblasts through its IRES*

We wanted to verify whether the potent eEF1A2-targeting drugs considered as hits in our screen, acted by stimulating IRES-mediated translation of utrophin A. For these experiments, we focused on the five hits that maximally upregulate eEF1A2 and utrophin A protein levels (Acarbose, Betaxolol, Labetalol, Pravastatin and Telbivudine), as determined by western blots (Table 4). Thus, we transfected cultured C2C12 myoblasts with a bicistronic construct, either the control CMV/βGAL/CAT or the CMV/βGAL/UtrA/CAT containing the utrophin A 5’UTR, and treated cells for 24 hours with vehicle control or one of the five top utrophin A- and eEF1A2-activating drugs. Reporter activity from cell lysates was assessed by using a CAT and β-gal ELISA reporter assay kit. The activity of the utrophin A IRES was determined by establishing a ratio of CAT to β-gal activity. Our data showed a significant ~1.35 to 2-Fold increase in CAT/β-gal ratios (P ≤0.05 and P ≤0.01) in which CAT levels increased and β-gal levels remained constant, thus demonstrating that the five top FDA-approved drugs did in fact activate utrophin A through IRES-mediated translation (Figure 27B). In addition, none of these drugs increased utrophin A mRNA levels, further suggesting activation of utrophin A through translational events (Figure 27C).
Table 4. Confirmation of eEF1A2 and utrophin A protein expression level increases in C2C12 cells, post FDA-approved drug treatment.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Utrophin A protein level (fold increase to veh ctl)</th>
<th>eeF1a2 protein level (fold increase to veh ctl)</th>
<th>Optimal Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acarbose</td>
<td>2.51</td>
<td>4.62</td>
<td>1µM</td>
</tr>
<tr>
<td>Betaxolol</td>
<td>2.13</td>
<td>4.26</td>
<td>5µM</td>
</tr>
<tr>
<td>Labetalol·HCl</td>
<td>3.25</td>
<td>1.41</td>
<td>1µM</td>
</tr>
<tr>
<td>Telbivudine</td>
<td>2.72</td>
<td>2.77</td>
<td>30µM</td>
</tr>
<tr>
<td>Pravastatin·Na</td>
<td>1.84</td>
<td>2.30</td>
<td>50nM</td>
</tr>
<tr>
<td>Olsalazine·Na</td>
<td>1.58</td>
<td>1.13</td>
<td>4µM</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>1.34</td>
<td>1.12</td>
<td>20nM</td>
</tr>
<tr>
<td>Nizatidine</td>
<td>1.22</td>
<td>no increase</td>
<td>1µM</td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td>1.17</td>
<td>no increase</td>
<td>10µM</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>no increase</td>
<td>1.44</td>
<td>5µM</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>no increase</td>
<td>1.30</td>
<td>5µM</td>
</tr>
</tbody>
</table>

*The drugs above the middle horizontal line are the five top drugs picked for further analysis in vitro and in vivo.*
Figure 28. Confirmation of eEF1A2 and utrophin A protein expression level increases in C2C12 cells, post FDA-approved drug treatment. Myoblasts were treated with 3 different concentrations of the 11 eEF1A2-activating drugs identified from the screen, including the concentration used in the high-throughput screen, for 24 hours. Protein extracts were subjected to western blot analysis. Western blots and quantifications of eEF1A2 and utrophin A protein levels normalized to ponceau. Table 4 summarizes the fold increase of both proteins to vehicle control (N=3-4). Error bars represent SEM, *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from vehicle control.
**Betaxolol and Pravastatin activate the utrophin A IRES in transgenic mice harboring the bicistronic reporter**

In a next series of experiments, we examined whether the five top drugs identified in the high-throughput screen cause upregulation of utrophin A via eEF1A2 thereby promoting IRES-mediated translation of utrophin A *in vivo*. To this end, we treated our IRES-transgenic mice (CMV/βGAL/UtrA/CAT) with one of the five drugs, or a vehicle control for a period of 7 days. After the treatment period, we observed that Pravastatin (2mg/kg) and Betaxolol (5mg/kg) increased endogenous levels of eEF1A2 (~2.5-fold) and utrophin A (~2 to 2.5-fold) in TA muscles (Figure 29 A, B). Note that these doses were selected in reference to past preclinical studies in mice and their clinical use in humans (Kim et al., 2016; Orbay et al., 2013). Western blot analyses of CAT and β-GAL were used to establish a ratio of CAT vs β-GAL activity to determine utrophin A IRES activity following treatment with Betaxolol or Pravastatin of transgenic mice. Despite some variability, Betaxolol and Pravastatin treatment resulted in a clear trend towards an increase in IRES reporter activity in muscles from these transgenic mice (~1.5 to 2-Fold; P = 0.3 and P = 0.6, respectively, Figure 29C). Furthermore, utrophin mRNA levels were unchanged post-treatment (Figure 29D), thus indicating that both drugs increased endogenous utrophin A protein levels *in vivo* by acting via the utrophin A IRES.
Figure 29. Pharmacological stimulation of utrophin A in transgenic bicistronic utrophin 5’UTR IRES harbouring mice. Transgenic mice were treated with Betaxolol (Bet) (5mg/kg), Pravastatin (Prava) (2mg/kg) or saline for 7 days (A, B) Western blots and quantification of eEF1A2 and utrophin A protein levels normalized to ponceau using protein extracts from TA muscles from the treated transgenic mice. C) Activation of 5’UTR utrophin IRES reporter construct after a 7-day treatment with Bet and Prava in transgenic mice. Representative western blots of CAT and β-gal protein levels from TA muscles from the treated transgenic mice. The levels of CAT/β-gal ratio of each drug normalized to vehicle control represents the IRES activity. D) Utrophin A mRNA levels in Bet, Prava or saline treated transgenic mouse TA muscles, standardized to 18S. N=8, error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001, vs vehicle control.
The eEF1A2-targeting drugs Betaxolol and Pravastatin enhance muscle strength of mdx mice

To establish whether Betaxolol and Pravastatin have therapeutic benefits in vivo, we treated 6-week old mdx and wild-type mice with either Pravastatin (2mg/kg), Betaxolol (5mg/kg) or saline for 4 weeks and analyzed the performance of the mice at the end of the treatment period. For this, mice were subjected to a digital gauge attached to a grid to detect forelimb and hindlimb grip strength. The weight of the mice was assessed throughout the 4-week treatment period and did not show any difference between drug and saline-treated mdx mice (Figure 30A). However, mdx mice treated for 4 weeks with Betaxolol showed significant improvements in forelimb grip strength (P < 0.05) as well as a trend towards an increase in hindlimb grip strength (P = 0.08) when normalizing for mouse weight (Figure 30B, C). The 4-week Pravastatin treatment of mdx mice induced striking ameliorations in forelimb grip strength (P < 0.01), rescuing it near wild-type levels, together with a significant increase in hindlimb grip strength (P < 0.01) with or without adjusting for mouse weight (Figure 30B, C). Overall, this demonstrates that both eEF1A2-targeting drugs improve muscle strength of dystrophic mice.

Betaxolol and Pravastatin increase sarcolemmal expression of utrophin A in mdx mice

After the 4-week treatment with Betaxolol, Pravastatin or vehicle control, TA muscles from wild-type and mdx-treated mice were dissected and cross-sectioned for further analyses. Since it is essential for utrophin A to localize to the sarcolemma in order to fully achieve its function (Miura and Jasmin, 2006), we examined utrophin A’s sarcolemmal localization in saline-, Betaxolol- and Pravastatin-treated TA muscles. Immunofluorescence analysis and quantification of positive fibers showing sarcolemmal utrophin A demonstrated that both Betaxolol and Pravastatin treatments nearly doubled the expression of utrophin A at the sarcolemma compared to vehicle control (Figure
**Figure 30. The effects of Betaxolol and Pravastatin treatment on muscle strength of mdx mice.** Mdx mice were treated with Betaxolol (Bet), Pravastatin (Prava) or saline for 4 weeks. **A** mdx and wild-type (WT) mouse body weight (g). **B** Forelimb and **C** Hindlimb grip strength analysis of WT, vehicle-treated, Bet- or Prava-treated mdx mice normalized or not normalized to body weight. N=8, error bars represent SEM. *P<0.05, **P<0.01 vs vehicle treated mdx mice or ## P<0.01, ###P<0.001 vs vehicle treated WT.
In addition, western blot analyses showed significant increases of both eEF1A2 (~1.5 and 1.8-Fold; P < 0.05 and P < 0.01) and utrophin A protein levels (~2.2 and 3.0-Fold; P < 0.05) in TA muscles from Betaxolol- and Pravastatin-treated mdx mice (Figure 31C and D). In agreement with a translational induction in utrophin A expression, qRT-PCR results showed no change in utrophin A mRNA levels in TA muscles from Betaxolol- or Pravastatin-treated mdx mice when compared to vehicle control (Figure 31E). This further indicates that the upregulation of utrophin A following treatment with either one of these two drugs occurs through translational events.

**Betaxolol and Pravastatin improve the integrity of dystrophic muscle fibers**

We next determined the effects of Betaxolol and Pravastatin on the morphology and integrity of dystrophic muscle fibers. To do so, we analyzed changes in central nucleation, a marker of muscle fiber regeneration (Briguet et al., 2004), by performing hematoxylin and eosin staining on cryostat sections, as well as intracellular IgM staining that reflects sarcolemmal damage. Our data revealed that both drugs decreased central nucleation (P < 0.05 and P < 0.01) (Figure 32A, C), and induced a striking ~3-Fold reduction in IgM infiltration into muscle fibers (P < 0.001) (Figure 32B, D). Altogether, these experiments show that increasing expression of utrophin A, through drug-induced activation of eEF1A2, attenuates the dystrophic pathology in mdx mice thereby illustrating the therapeutic potential of these drugs for treating DMD patients.

**Pravastatin treatment of eEF1A2-null mice fails to upregulate utrophin A expression**

To determine whether utrophin A upregulation by Pravastatin is directly dependent on eEF1A2, we performed daily treatments of eEF1A2-null and wild-type mice with either Pravastatin or vehicle control for 5 days. eEF1A2 mice, commonly referred to as wasted mice,
Figure 31. Increase of sarcolemmal localization and protein levels of utrophin A by Betaxolol and Pravastatin treatment in mdx mice. A) Representative examples of cross-sections obtained from TA muscles of wild-type (WT) mice and mdx mice treated with Betaxolol (Bet), Pravastatin (Prava) or vehicle control (saline), were immunostained with utrophin A (UTR-A) antibody. Scale bars, 50 mm. B) Quantification of sarcolemmal utrophin A positive fibers to total muscle fibers. C, D) Western blots and quantification of eEF1A2 and utrophin A protein levels normalized to ponceau using protein extracts from TA muscles from the treated mdx mice. E) Utrophin A mRNA levels in Bet-, Prava- or saline-treated mdx mouse TA muscles, standardized to 18S. N=7-8, error bars represent SEM. *P < 0.05, **P < 0.01, vs vehicle control.
Figure 32. Morphological features of mdx muscle fibers treated with Betaxolol and Pravastatin. A) Representative examples of cross-sections of TA muscles from wild-type (WT) and mdx mice treated with Betaxolol (Bet), Pravastatin (Prava) or with vehicle (saline) that were stained using hematoxylin and eosin. C) Percentage of central nucleation in TA muscle fibers. B) Representative examples of cross-sections of TA muscles from WT mice and mdx mice treated with Bet, Prava or vehicle that were immunostained with goat-anti-mouse IgM Alexa 594. D) Quantification of IgM positive fibers vs total muscle fibers. N=8, error bars represent SEM, *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from respective control. Scale bars, 50 mm.
possess a naturally occurring ~16kb deletion that eliminates the first non-coding exon and regulatory promoter elements of the gene encoding eEF1A2, leading to complete ablation of its expression (Chambers et al., 1998; Newbery et al., 2005; Shultz et al., 1982). eEF1A1 is highly expressed in neuronal, cardiac and skeletal tissues during embryonic development but its expression gradually declines after birth until it becomes undetectable by day 21. By contrast, eEF1A2 expression in these tissues increases starting shortly before birth until it reaches a plateau by day 21 (Khalyfa et al., 2001). Therefore, wasted mice show striking neuromuscular deficits starting at day 21 which leads to their death at approximately day 28 (Abbott et al., 2009; Chambers et al., 1998). Accordingly, we treated wasted and wild-type mice with Pravastatin starting at day 20 for five days.

Our data demonstrate that a short five-day treatment of wild-type mice with Pravastatin was sufficient to induce a ~ 2.7-fold increase (P < 0.05) in the levels of endogenous utrophin A compared to saline control (Figure 33A and C). Remarkably, and in complete agreement with our working hypothesis, Pravastatin treatment of eEF1A2-null mice did not cause an increase in utrophin A expression (Figure 33B and C). These data show that Pravastatin-mediated upregulation of utrophin A is dependent on eEF1A2.
Figure 33. Pravastatin does not induce a utrophin A increase in eEF1A2-null mice. eEF1A2-null mice (wasted mice – wst) and wild-type mice (WT) were treated with Pravastatin (Prava) (2mg/kg) or saline for 5 days A and B) Western blots of utrophin A protein levels and ponceau, using protein extracts from TA muscles from wild-type mice and wasted mice treated with pravastatin or vehicle control. C) Quantification of utrophin A protein levels normalized to ponceau in treated WT and wasted mice. N=3-6, error bars represent SEM, *P < 0.05, significantly different from respective control.
Discussion

In the present study, we set-out to: 1) examine whether eEF1A2 regulates utrophin A expression through IRES-mediated translation; and 2) identify and characterize FDA-approved drugs that might offer benefits to dystrophic muscles by acting through this pathway. Through a series of complementary studies, we demonstrate that eEF1A2 directly regulates endogenous utrophin A protein expression, via IRES-dependent translation. Moreover, we designed a high-throughput screen targeting eEF1A2 and identified seven classes of FDA-approved drugs able to activate IRES-dependent translation of utrophin A. Further investigation of two of these drugs, Betaxolol and Pravastatin, revealed their ability to activate this pathway in vivo and to improve the dystrophic phenotype of mdx mice. Taken together, our findings illustrate the feasibility of targeting eEF1A2/IRES-mediated translation of utrophin A with repurposed drugs as a therapeutic approach for treating DMD.

In this work, we compared endogenous levels in both slow soleus and fast EDL muscles of eEF1A2 in mdx vs wild-type mice. Previous studies have shown that utrophin A mRNA and protein are more abundant in slow muscles compared to fast ones, and that this increased expression is in part due to an enrichment of utrophin A in extrasynaptic regions (Gramolini et al., 2001). Here, we did not detect a difference in endogenous eEF1A2 levels between fast or slow skeletal muscles indicating that eEF1A2 does not play a significant role in differentially regulating utrophin A expression in fast versus slow muscles. Moreover, we observed that eEF1A2 is similarly expressed in muscles of wild-type versus mdx mice. This latter finding is important as it suggests that overexpression of eEF1A2 in mdx muscles may indeed be a viable therapeutic approach for increasing endogenous utrophin A levels in dystrophic muscle fibers as we have shown here in proof-of-principle studies.
From our in-cell ELISA-based high-throughput drug screen that contained 262 FDA-approved drugs, we identified 11 drugs that activated both eEF1A2 and utrophin A. We noted that some of these drugs have common functions such as anti-diabetic, anti-peptic ulcer, lowering cholesterol and beta-adrenergic agents. This is interesting and consistent with the fact that several anti-diabetic drugs and statins (cholesterol-lowering drugs) have been shown to improve dystrophic muscles (Al-Rewashdy et al., 2015; Hafner et al., 2016; Jahnke et al., 2012; Ljubicic et al., 2011; Pauly et al., 2012; Péladeau et al., 2016; Whitehead et al., 2016). In addition, specific beta-adrenergic blockers such as Carvedilol were shown to improve cardiac function in DMD patients (Rhodes et al., 2008). Nonetheless, a recent study showed that the statin simvastatin induced beneficial effects in mdx mice in part by reducing oxidative stress but without effects on utrophin A expression. It is important to note that simvastatin was part of the 262 drugs that were tested in our current study, and that our results thus confirm its inability to increase utrophin A protein levels. While several drugs play common roles, they may still activate distinct pathways explaining why only some statins affect utrophin A levels.

Following confirmation of the ability of these drugs to induce eEF1A2 and utrophin A expression, we decided to further investigate the five top drugs for their capacity to increase IRES-mediated translation of utrophin A. Using a cell culture system with myoblasts, we demonstrate that these five drugs all stimulate the activity of the utrophin A 5’UTR IRES reporter construct without causing any changes in utrophin A mRNA levels. This indicates that indeed these drugs promote IRES-dependent translation of utrophin A.

Based on a 7-day drug treatment of our transgenic mice harbouring the bicistronic reporter construct containing the utrophin 5’UTR, we observed that the beta-androgenic blocking agent Betaxolol and the cholesterol-lowering drug Pravastatin were the most potent activators of both
eEF1A2 and utrophin through its 5’UTR IRES in vivo. Furthermore, a 4-week treatment of mdx mice with these drugs elicited significant improvements of the dystrophic phenotype that include increases in muscle strength, amelioration of muscle fiber morphology and sarcolemmal integrity. In order to confirm that utrophin A upregulation was dependent upon eEF1A2 activity, we also treated eEF1A2-null (wasted) mice with Pravastatin and show that the utrophin A upregulation, as seen in treated wild-type mice, was completely abolished in muscles from wasted mice. Altogether, these experiments show that the ITAF eEF1A2 is able to regulate utrophin A IRES-dependent translation and that drugs such as Betaxolol or Pravastatin can modulate utrophin A levels vis eEF1A2 thereby revealing their potential as relevant repurposed agents for treating DMD.

Our current findings showing the role of eEF1A2 as an ITAF are in agreement with the recent demonstration that another elongation factor, eEF2, acts as an ITAF to regulate IRES-mediated translation of XIAP and FGF2 mRNAs (Argüelles et al., 2014). Currently, it is unknown how elongation factors could act as ITAFs to regulate IRES-mediated translation of specific mRNAs. However, it is possible that they may interact with tRNA-like structural elements found in IRES’s given the main role of elongation factors in shuttling aminoacylated tRNAs to the ribosomal A site, enabling continuation of protein synthesis (Browne and Proud, 2002; Merrick, 1992; Moldave, 1985). In this context, it seems important to determine whether eEF1A2 also regulates the activity of other IRES’s, including the recently described IRES found in dystrophin mRNAs (Wein et al., 2014).

There are two isoforms of eEF1A: eEF1A1 and eEF1A2, with both isoforms playing similar roles in translation elongation (Ann et al., 1991; Knudsen et al., 1993b; Lund et al., 1996). Over the past few years however, a number of non-canonical roles have been identified for eEF1A, some
of which appear to be specific to either 1A1 and 1A2 variants. For example, in terminally-differentiated myotubes, eEF1A1 was shown to promote apoptosis whereas eEF1A2 played an anti-apoptotic role (Ruest et al., 2002), suggesting clear distinct roles for the two isoforms (Duanmin et al., 2013; Lee and Surh, 2009; Xu et al., 2013). Furthermore, eEF1A1 and eEF1A2 are differentially expressed in tissues. In contrast with eEF1A1 that is generally expressed ubiquitously, eEF1A2 is preferentially expressed in skeletal muscle, heart and brain tissues (Knudsen et al., 1993a; Lee et al., 1992), suggesting that eEF1a2 plays a distinct role in muscle.

In summary, our study shows that eEF1A2 directly regulates IRES-mediated translation of utrophin A. Moreover, our high-throughput drug screen identified specific classes of FDA-approved drugs able to increase utrophin A through eEF1A2 and IRES-dependent translation with at least two of them being able to significantly improve the dystrophic phenotype of mdx mice. Our work thus reveals the therapeutic relevance of eEF1A2 as a target for pharmacological interventions in DMD and shows the feasibility of using repurposed drugs to activate this pathway for treating this neuromuscular disorder DMD.
Materials and Methods

**In-Cell ELISA High-throughput drug screen**

An ELISA-based high-throughput limited drug screen was designed using an In-Cell ELISA Colorimetric Detection Kit (Thermo Fisher Scientific, Massachusetts, USA). For this, a total of 262 FDA-approved drugs were aliquoted in 384-well microplates derived from the Screenwell FDA-approved drug library V2 (Enzo Life Sciences – Cederlane – Ontario, Canada). C2C12 myoblasts were grown in each well and treated with these drugs or vehicle control for 24 hours. Following treatment, antibodies targeting eEF1A2 (1:1000, provided by Dr. Abbott) or utrophin A (1:500; Novocastra, Leica biosystems, Concord, ON, Canada) and HRP-conjugated IgG secondary antibodies (Jackson Immuno Research, Bar Harbor, USA) were used to detect protein expression levels. Absorbance levels were determined with a Synergy H1 microplate reader. Note that the absorbance levels are standardized to total cell number by using a whole-cell stain in order to control for variation in cell proliferation.

Confirmation experiments were performed by treating C2C12 myoblasts with 11 drugs: Acarbose, Betaxolol, Labetalol-HCl, Telbivudine, Pravastatin-Na, Olsalazine-Na, Lovastatin, Nizatidine, Propylthiouracil, Hydrochlorothiazide and Rosiglitazone at concentrations indicated (Figure 28). 24-hours later, cells were harvested in urea buffer and subjected to western blot analysis.

**Reporter assays**

C2C12 skeletal muscle myoblasts were transfected with one bicistronic construct, either the control CMV/βGAL/CAT or the CMV/βGAL/UtrA/CAT containing the utrophin A 5’UTR. The next day, cells were treated with the various drugs or vehicle control for 24 hours. Reporter activity
from cell lysates was assessed by using the CAT and β-gal ELISA reporter assay kits (Roche, QC, Canada) and a Synergy H1 microplate reader. The activity of utrophin A IRES was determined by establishing a ratio of CAT to β-gal activity. It is important to note that extensive control experiments have been previously performed to ensure that the bicistronic mRNA does not undergo aberrant splicing, while also showing that the utrophin A 5’-UTR does not contain cryptic promoter activity (Miura et al., 2005, 2010).

For in vivo analysis, proteins from transgenic mouse TA muscles were extracted with reporter lysis buffer (Promega, Wisconsin, USA) as previously described (Miura et al., 2010). The protein concentration was determined using the bicinchoninic acid (BCA) assay. Protein samples were diluted to a final concentration of 4 mg/ml prior to performing the reporter assays. βGAL enzymatic assays were performed using the β-GAL enzyme assay system as recommended by the manufacturer (Promega). To measure CAT activity, we analyzed the conversion of chloramphenicol to butyryl-chloramphenicol by incorporation of [14C] butyryl coenzyme A (Miura et al., 2010). Background levels for the reporter assay were determined by analyzing reporter activity in tissues from mice not harboring a transgene.

Animal strains and experiments

In vivo experiments were performed using C57BL/10ScSn-Dmdmdx/J (mdx) mice, C57BL/10 (wild-type) mice (The Jackson Laboratory, Bar Harbor, USA), B6C3Fe a/a-Eef1a2wsf/J mice and B6C3Fe a/a wild-type mice (The Jackson Laboratory - JAX stock #000182), as well as the transgenic mice harboring either a CMV/bGAL/CAT or a CMV/bGAL/UtrA/CAT bicistronic reporter transgene (Miura et al., 2010). These mice were maintained in the Animal Care and Veterinary Service at the University of Ottawa.
Direct plasmid injection/electrotransfer was performed in TA muscles as previously described (Ravel-Chapuis et al., 2012). Briefly, eEF1A2 expression constructs (MYC-HIS360-eEF1A2-pcDNA) or pcDNA3.1 control was electroporated into either one of the two TA muscles while the animals were under anesthesia. Seven days after electrotransfer, mice were euthanized, and TA muscles were dissected for further analysis.

**In vivo drug treatment**

CMV/bGAL/UtrA/CAT transgenic mice were treated daily with either Betaxolol (5mg/kg) (Selleckchem, Houston, Texas, USA), Pravastatin-Na (2mg/kg) (Santa-Cruz, Dallas, Texas, USA) or vehicle control (saline) for 7-days by intraperitoneal injection (IP). B6C3Fe a/a-Eef1a2<sup>wt</sup>/J mice and B6C3Fe a/a wild-type were treated with Pravastatin-Na (2mg/kg) (Santa-Cruz) or vehicle control (saline) for 5-days by IP injection. A shorter treatment time was performed with these mice due to their short life expectancy of approximately 28 days (Chambers et al., 1998). Both drugs were dissolved in sterile saline prior to each treatment. Six-week-old mdx or wild-type mice were treated daily with Betaxolol (5mg/kg/day), Pravastatin-Na (2mg/kg/day) or vehicle control (saline) by IP injection for 4-weeks, a treatment period regularly performed in our laboratory (Al-Rewashdy et al., 2015; Ljubicic et al., 2011; Péladeau et al., 2016, 2018). Muscles were then dissected from euthanized mice, and either flash frozen in liquid nitrogen or embedded in Optimum Cutting Temperature compound (OCT) and frozen in melting isopentane cooled with liquid nitrogen.
**Forelimb grip strength**

Forelimb grip strength analysis was performed on the final day of drug treatments and was evaluated with the use of a digital force gauge, Chatillon DFE II (Columbus Instruments, Columbus, USA) and a grid. The mice were first acclimatized to the work area for 30 minutes. They were then permitted to grip the grid attached to the digital gauge and pulled horizontally away from the bar in a constant motion, until release of the grid. The process was repeated six times per mouse accompanied by a 30 second rest time between each measurement. The value of the maximal peak force was recorded (gF). The grip strength measurements were conducted by the same investigator in order to limit variability and were performed in a random order. The investigator performing the measurements was blinded as to the treatment group of each individual mouse upon testing.

**Western blotting**

Determination of protein expression in different tissue samples was analyzed by western blotting as previously described (Péladeau et al., 2016). C2C12 skeletal muscle cells or mouse muscle tissues were homogenized in urea buffer supplemented with protease inhibitor (Roche). A total of 5-20 μg of protein extracts were resolved on either a 7% SDS-PAGE gel for utrophin A and eEF1a2 analyses, or 10% SDS PAGE gels for CAT and β-Gal analyses. Proteins were transferred overnight at 4°C onto nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada, 0.45μm). Membranes were then stained with Ponceau to confirm equal loading and blocked as described previously (Péladeau et al., 2016). Membranes were incubated with primary antibodies directed against utrophin A (1:500; Novocastra), eEF1A2 (1:1000, provided by Dr. Abbott and Abcam, Toronto, ON, Canada), CAT (1:1000, Abcam), β-Gal (1:1000, Abcam) and β-actin
(1:10000; Santa Cruz, Dallas, Texas, USA). Blots were probed with appropriate HRP-conjugated IgG secondary antibodies (Jackson ImmunoResearch). Protein detection was performed by using ECL reagent (Perkin Elmer, Waltham, MA, USA). The films were quantified using ImageJ (NIH version 1.0) and/or Image Lab.

**RNA isolation and qRT-PCR**

RNA isolation, reverse transcription and qRT-PCR were performed using methods previously described (Miura et al., 2010; Péladeau et al., 2016). Briefly, total RNA was isolated from muscle tissues from wild-type and mdx mice using TRIzol reagent (Invitrogen, Carlsbad, California, USA). Real-time quantitative RT-PCR was carried out with primers to amplify utrophin A and eEF1A2 mRNA (Miura et al., 2010; Péladeau et al., 2016). 18S ribosomal RNA was used as a housekeeping gene.

**Immunofluorescence**

Immunofluorescence experiments were performed as previously described (Péladeau et al., 2016) al.) using TA muscle cross-sections from wild-type or mdx mice. Cross-sections were prepared for immunofluorescence analysis using the M.O.M Immunodetection kit (Vector Laboratories, Burlington, ON, Canada). The sections were incubated with primary antibody directed against utrophin A (1:200, NCL-DRP2, Novacstra) and Texas Red-conjugated Streptavidin antibody (1:500; Vector laboratories) or against a FITC-conjugated IgM anti-mouse secondary antibody (1:400; Sigma-Aldrich, Oakville, Canada). All muscle tissue sections were co-stained with a rabbit laminin antibody (1:800; Sigma-Aldrich, Oakville, Canada) and goat anti-rabbit Alexa Fluor 488 IgG secondary antibody (1:500; ThermoFisher Scientific, Massachusetts,
USA) to highlight the sarcolemma. The slides were mounted with Vectashield containing DAPI staining (Vector Laboratories, Burlington, ON, Canada) and visualized using a Zeiss Axioskop-2 microscope. Quantification was accomplished using Image J (NIH version 1.0).

TA muscle cross-sections were stained with Hematoxylin and Eosin dyes and processed as previously described (Péladeau et al., 2016). The sections were mounted using Permount (Fisher Scientific, Ottawa, Canada) and visualized using an epifluorescent EVOS FLAuto2 inverted microscope. Percentage of central nucleation was determined by counting the total number of muscle fibers and the number of centrally nucleated muscle fibers from 6-8 cross-sectional views using the Northern Eclipse Software (NES, EMPIX Imaging, Mississauga, Ontario, Canada).

**Statistical analysis**

The data were analyzed using two-tailed student t-tests and one-way ANOVAs (Analysis of Variance) with Bonferroni post-hoc tests. Error bars represent standard error of the mean (SEM). Statistical analysis was done with Graph Pad prism 6 (Prism Software, La Jolla, CA, USA). The level of significance was set at $P \leq 0.05$. 
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Chapter 5 – General Discussion
General Discussion

5.0 Summary

Despite the many studies in various phases of clinical trials being developed in hopes of finding a treatment for DMD, there is a critical need for the development of therapies with high efficiency, ease of administration, and with wide applicability that can be quickly brought to the market. Increasing evidence suggests that pharmacological upregulation of utrophin A protein in dystrophic muscles is a promising and safe therapeutic strategy for the treatment of DMD. This work was based on the hypothesis that repurposing FDA-approved drugs activating utrophin A, will be an efficient approach in rapidly bringing new therapeutic interventions for DMD. This thesis is comprised of three manuscripts forming a collection of work that exploits the availability of FDA-approved drugs or compounds in clinical trials for other diseases and describes their ability to stimulate utrophin A through post-transcriptional and translational events, while also having beneficial effects in dystrophic muscles.

The first manuscript (chapter 2) was based on previous work from our lab demonstrating the ability of the anti-coagulant drug, Heparin, to induce stabilization of utrophin A transcripts by phosphorylating KSRP through the activation of p38 MAPK (Amirouche et al., 2013). In light of this, we showed that a long-term treatment with the FDA-approved drug Heparin in mdx mice activated this key pathway and resulted in a significant increase of utrophin A expression in the TA and diaphragm muscles. More importantly, this treatment improved muscle fiber integrity and morphology of mdx mice. In this work we also performed a combinatorial treatment with the utrophin A post-transcriptional activator Heparin and transcriptional activator AICAR. We demonstrated that the combination of both drugs activating utrophin A through distinct pathways,
promotes an additive effect on utrophin A expression levels in the TA, diaphragm and the heart. The second manuscript (chapter 3) revealed the valuable effects of a second p38 MAPK activator, the FDA-approved NSAID Celecoxib, in mdx mice muscles. Similarly to Heparin, Celecoxib ameliorated muscle fiber integrity but also improved muscle function, decreased immune cell infiltration and was accompanied by an increase of utrophin A in the TA, diaphragm and in the heart of mdx mice. These two studies suggest that the use of p38 MAPK activating drugs to increase utrophin A in dystrophic muscles is a promising therapeutic approach for DMD. The final manuscript (chapter 4) describes the potential of targeting the ITAF, eEF1A2, to promote IRES-dependent translation of utrophin A in skeletal muscles. By performing a high-throughput FDA-approved drug screen, we identified 11 drugs that activated both eEF1A2 and utrophin A protein levels in skeletal muscle cells, which could be subdivided in seven classes of drugs. Two of these drugs, Betaxolol and Pravastatin activated a bicistronic reporter construct containing utrophin A’s 5’UTR IRES site \textit{in vitro} and \textit{in vivo} and significantly improved the dystrophic phenotype of mdx mice. This data reveals the advantages of targeting eEF1A2 in dystrophic muscles to induce IRES-dependent translation and shows a new approach to identify FDA-approved drugs for the treatment of DMD. Collectively, these articles indicate that pharmacological stimulation of utrophin A using FDA-approved drugs may be a promising therapy for DMD and that combinatorial treatment could indeed be an effective strategy to promote a synergistic effect in dystrophic muscles (see model in figure 34).
Figure 34. Utrophin A upregulation by FDA-approved drugs in dystrophic muscles.

A) AICAR activates AMPK which phosphorylate PGC-1α in dystrophic muscles. Coactivation, with PGC-1α and other transcription factors (such as PPARβ/δ and GABP) stimulate utrophin A transcription. B) Betaxolol and Pravastatin increase eEF1A2 expression levels in mdx mouse muscles. eEF1A2 acts as an ITAF and binds utrophin A mRNA at it’s 5’UTR IRES site and permits recruitment of the ribosome to utrophin A mRNA’s 5’UTR which promotes cap-independent utrophin A translation. C) P38 stimulation by Celecoxib activates HuR which may interact with utrophin A’s IRES site at its 5’UTR to induce cap-independent translation of utrophin A. D) Heparin induces a decrease in the functional availability of KSRP, which is involved in ARE-mediated decay of utrophin A transcripts. The reduction in the functional availability of KSRP is promoted by an increase in its phosphorylation which mediates its sequestration by the regulatory protein 14-3-3; or a decrease in its expression, which in turn results in an increase of utrophin A expression. Combinatorial stimulation of these distinct pathways may have an additive effect on utrophin A expression levels as we demonstrate with AICAR and heparin treatment in chapter 2.
5.1 P38-Activating Drugs, Heparin and Celecoxib, Stimulate Utrophin A in Mdx Mouse Muscles

Recently our lab demonstrated the novel role of p38 MAPK in mediating the post-transcriptional regulation of utrophin A by decreasing the ability of KSRP to interact with the ARE in utrophin’s 3’ UTR which causes an increase in utrophin A mRNA and protein levels (Amirouche et al., 2013). More specifically, p38 MAPK phosphorylates KSRP which creates a functional binding site for the molecular chaperone 14-3-3. In consequence, p38 MAPK activation induces sequestration of KSRP which diminishes its ability to interact with the exosome to promote rapid mRNA decay of utrophin A (Amirouche et al., 2013). This study also demonstrated that a short 10-day treatment with the p38-MAPK activating drug Heparin could induce post-transcriptional activation of utrophin A in mdx skeletal muscles (Amirouche et al., 2013). As described in chapter 2, our work expanded on these findings and showed that a longer treatment with Heparin (4-weeks) activated this key pathway resulting in approximately a 2-Fold upregulation of utrophin A transcript and protein levels in skeletal muscles and diaphragm. Heparin treatment also rescued utrophin A localization and DAPC formation at the sarcolemma of the dystrophic muscle fibers, which is required to compensate for the lack of dystrophin (Amenta et al., 2011; Chaubourt et al., 1999; Tinsley et al., 1998). In our second study (chapter 3), we used an FDA-approved drug called Celecoxib, recently showed to activate p38 MAPK in human and mouse neuronal cells (Farooq et al., 2013), to treat myotubes for 24-hours and mdx mice for 4-weeks. We demonstrated that Celecoxib increases utrophin A expression levels by ~2.5-Fold in skeletal myotubes as well as a ~2-Fold augmentation in TA, diaphragm and heart of mdx mice. These results are encouraging since it has been shown that a 2-Fold transgenic overexpression of utrophin A in mdx mice was sufficient to elicit full recovery of mechanical functions of dystrophic muscle (Fisher et al., 2001;
These important findings dictate that pharmacological activation of p38 MAPK may be an effective method to increase utrophin A in dystrophic muscles as a therapeutic approach for DMD.

There have been many discrepancies between studies involving the effects of activating p38 MAPK in dystrophic muscles. Although p38 MAPK is well known for its crucial role in immune and inflammatory response (Cuenda and Rousseau, 2007), increased evidence indicates that its activation modulates many other cellular aspects of the physiology of the cell including cell cycle, cytoskeleton remodelling and myogenic development (Cuenda and Cohen, 1999; Cuenda and Rousseau, 2007; Li et al., 2000; Wu et al., 2000; Zetser et al., 1999). For instance, this kinase induces transcription of myocyte enhancer factor-2 (MEF2) members involved in myofiber differentiation and promotes the stabilization of muscle-specific regulators (myogenin and p21) which play distinct roles, in transition of myoblasts to myotubes (Briata et al., 2005; Gillespie et al., 2009; Lluis et al., 2005; Perdiguerio et al., 2007; Rampalli et al., 2007; Serrano et al., 2008). Interestingly, a study from Shi et al. discussed the importance of p38 MAPK in mdx mice. In fact, they report that mdx mice lacking the p38 MAPK negative regulator, dual specificity phosphatase-10 (Dusp10), showed improved regenerative myogenesis as well as amelioration of the dystrophic phenotype (Shi et al., 2013). Thus, based on our new findings, it would be of great interest to determine if the improvement of the dystrophic phenotype observed here was in part due to an increase in utrophin A expression levels. However, in contrast, mdx mice treated with the p38 inhibitor SB203580 for 1h increased the survival of mdx myofibers after oxidative stress (Smythe and Forwood, 2012), which may be at least partially due to the ability of the p38α isoform to induce activation of the pro-death effector Bax (Wissing et al., 2014). Further discrepancies

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between p38 MAPK isoforms has been reported where p38γ, but not p38α or p38β, is required for endurance exercise-induced mitochondrial biogenesis and angiogenesis (Pogozelski et al., 2009).

Overall, the discrepancies shown in these studies seem to be based on isoform specific p38 activation and upstream activators of p38 MAPK. Thus, it would be important to determine which isoform is activated by Heparin and Celecoxib to better understand which isoform can activate utrophin A and thereby be beneficial for dystrophic muscles. Nevertheless, our work describes the ability of two p38-activating drugs, Heparin and Celecoxib, to increase utrophin A in multiple dystrophic muscles of mdx mice and their potential as pharmacological treatments for DMD.

5.2 Celecoxib Activates Utrophin A Through Translational Regulation

Although our study and others clearly demonstrate that Celecoxib activates p38 MAPK (Farooq et al., 2013) resulting in upregulation of utrophin A, our data suggest that this is not through the phosphorylation of KSRP. In contrast to Heparin, Celecoxib only increased protein levels of utrophin A in mdx mouse muscles and was unable to modulate utrophin A transcripts. Thus, a possible mechanism is that Celecoxib treatment activates IRES-dependent translation of utrophin A through the regulation of key signaling proteins. One such protein is the RNA-binding protein HuR which was previously shown to promote translation of target mRNAs by interacting with IRES sequences located in the 5’UTR (Durie et al., 2011; Lin et al., 2015; Srikantan et al., 2012). In addition, HuR can also be activated by p38 MAPK (Kim et al., 2010; Lafarga et al., 2009; Myer et al., 1997; Vreeland et al., 2014). In this context, Farooq et al. showed that HuR is required for the SMN protein increase seen in Celecoxib-treated human and mouse neuronal cell (Farooq et al., 2013). Interestingly, we observed that Celecoxib induced an increase in HuR protein levels in both TA and diaphragm muscles. Collectively, these data suggest that Celecoxib
stimulates HuR protein expression in muscle, which in turn may facilitate interaction of HuR with the utrophin A IRES causing increased translation of utrophin A. Additional work is necessary to examine the link between p38 activation, HuR and translation of utrophin A.

5.3 TLR Activation of P38 MAPK: A Potential Target to Increase Utrophin A

One question that has not yet been investigated is the identity of signaling molecules that act upstream of p38 following Heparin and Celecoxib treatment. One possible candidate could be TLRs that have been shown to play a role in activating p38 MAPK (Senn, 2006; Zbinden-Foncea et al., 2012). TLRs are transmembrane receptor proteins that play an important role in the innate immune system. There are thirteen known TLRs of which seven have been shown to be expressed in skeletal muscles (Henriques-Pons, 2014; Rawat et al., 2010; Reyna et al., 2008; Senn, 2006, Tournadre, 2010). TLR1, 2, 3, 4, 7, 8 and 9 are expressed at distinct levels in different skeletal muscles of mdx mice. It is also reported that slow-twitch muscles such as the soleus, show increased levels of TLRs compared to diaphragm and gastrocnemius (Henriques-Pons et al., 2014). Interestingly, Heparin has been shown to activate p38 MAPK through TLR2 and TLR4 in skeletal muscle wild-type mice by increasing extracellular levels of non-esterified fatty acids (Zbinden-Foncea et al., 2012). In addition, healthy patients treated with a single dose of Celecoxib, produced tumor necrosis factor alpha (TNF) in response to TLR4 and TLR8 ligand lipopolysaccharide (LPS) drawn from whole blood samples (Page et al., 2010). This suggests that Heparin and Celecoxib could both stimulate TLRs, which may activate p38 MAPK resulting in an increase in utrophin A expression in dystrophic muscles. There are a few discrepancies between studies investigating TLRs in dystrophic muscles. In fact, researchers have shown that the ablation of TLR4 or its adaptor protein myd88, in mdx mice, promotes beneficial effects to their muscle (Giordano et al.,
However, increasing evidence suggests that TLRs are key players in the regulation of macrophages during regenerative myogenesis in response to muscle injury (Hindi and Kumar, 2016). Therefore, additional work is required to have a better understanding of the therapeutic potential of TLRs in dystrophic muscles and to investigate their potential role in activating utrophin A expression.

5.4 Heparin and Celecoxib as Potential Treatments for DMD

Our work demonstrating the ability of Heparin and Celecoxib to increase utrophin A expression levels in mdx muscle fibers was accompanied by a substantial improvement of the dystrophic phenotype. In fact, our work leads us to believe that Heparin and Celecoxib may be potential drugs for the treatment of DMD for the following reasons: 1) Both drugs upregulated utrophin A in key muscles including the diaphragm and the heart. 2) Heparin and Celecoxib improved sarcolemmal integrity and morphological features of the muscle fibers. 3) These drugs also promoted a fiber type switch to slow fibers.

First, as mentioned above, Heparin and Celecoxib increased expression levels of utrophin A in key muscles such as the TA, the diaphragm and the heart following a 4-week treatment. This is important because the main cause of death for DMD patients is respiratory and cardiac failure (Bach et al., 1987; Guiraud et al., 2015a). In addition, the most severely affected muscle in mdx mice is the diaphragm (Stedman et al., 1991), thus, this suggests that Heparin and Celecoxib may potentially have the ability to upregulate utrophin A and hence have beneficial effects, in a more severe model. Furthermore, one of the main hurdles faced by many when developing a treatment for DMD, such as exon skipping, premature stop codon read through as well as gene therapy, is the adequate uptake or repair in the heart due to its anatomic location and function (Goyenvalle
and Davies, 2011; Hoffman et al., 2011; Yue et al., 2003). We report that pharmacological treatment of mdx mice with Celecoxib or combinatorial treatment of Heparin and AICAR can indeed induce utrophin A expression in the heart. Thus, treatments with these drugs could potentially reduce cardiac complications in DMD patients since a recent study has shown that utrophin upregulation in the heart improves cardiac function in severely affected D2/mdx mice (Kennedy et al., 2018).

Next, we show that Heparin and Celecoxib improve key features of the dystrophic phenotype in mdx mice. Both drugs strikingly decreased penetration of IgM within the muscle fiber. Due to a loss of mechanical reinforcement from dystrophin, the sarcolemmal integrity is compromised and weakened in dystrophic muscles, permitting influx of molecules, including serum antibodies, to enter the muscle (Duncan, 1989; Straub et al., 1997). Our data demonstrate that Heparin and Celecoxib improved sarcolemmal integrity due to the restoration of utrophin A and DAPC members at the sarcolemma of the muscle fibers. In addition, mdx mice treated with either Heparin or Celecoxib elicited morphological changes of their muscle fibers. In fact, both treatments induced a shift towards larger fibers. Heparin treatment also showed a decrease in fiber size variability as well as reduced centrally nucleated muscle fibers. Studies have shown that abnormal fiber size distribution is a hallmark of dystrophic muscles due to the ongoing cycles of degeneration and regeneration of the muscle fibers (Briguet et al., 2004; Torres and Duchen, 1987), while central nucleation is a marker of muscle damage and regenerating fibers (Briguet et al., 2004). Although the exact mechanisms underlying these morphological changes are not understood, if TLRs are activated as hypothesized and suggested previously (Zbinden-Foncea et al., 2012), it may be a result of interleukin 6 (IL-6) production in response to TLR activation (Machida et al., 2006) which can promote muscle fiber hypertrophy (Pillon et al., 2013; Serrano
et al., 2008). Our results also demonstrated that Celecoxib treatment improved the grip strength of mdx mice similarly to levels observed in WT control mice. Since grip strength of mdx mice treated with Heparin was not assessed, it would be important to functionally test mdx mice following a 4-week Heparin treatment to determine if Heparin improves grip strength as observed with celecoxib treatment. Collectively, these findings reveal the ability of Heparin and Celecoxib to improve many features of the dystrophic phenotype of mdx mice.

Finally, Heparin and Celecoxib treatments induced a shift towards a slow phenotype. In fact, both drugs stimulated the expression of slow fiber type markers including, slow MHC I and MHC IIa as well as slow oxidative phosphorylation markers (complex IV - cytochrome C oxidase, and complex V - ATP-synthase). Many studies have led to the idea that promotion of a slower, more oxidative phenotype, which causes increased utrophin A, is a viable therapeutic strategy for DMD (Al-Rewashdy et al., 2015; Chakkalakal et al., 2003; Chalkiadaki et al., 2014; Ljubicic et al., 2011, 2014; Miura et al., 2009). In fact, dystrophic slow muscle fibers show reduced damage compared to fast muscles, which may be partially due to increased endogenous utrophin A expression in slow muscle fibers, thus, creating a protective effect on the muscle fibers during contractions (Consolino and Brooks, 2004; Gramolini et al., 2001; Moens et al., 1993; Webster et al., 1988). It is unclear how Heparin and Celecoxib promote the transition in fiber type and metabolic profile in mdx mice. P38γ was shown to be required for endurance exercise-induced mitochondrial biogenesis and angiogenesis, however, studies show that it is unable to induce fiber-type switch transformation (Pogozelski et al., 2009), therefore, the fiber type switch seen in Heparin and Celecoxib treated mdx muscles may not be dependent on p38 activation. Additional studies need to be performed in order to determine how both drugs might promote a switch towards a slow oxidative phenotype in dystrophic muscles.
In summary, these data report that Heparin and Celecoxib can increase utrophin A in skeletal muscles as well as the diaphragm and the heart. This results in the improvement of many features of the dystrophic phenotype, thus revealing the potential of Heparin and Celecoxib as a therapy for DMD.

5.5 Heparin and Celecoxib are Multifunctional Drugs

Although Heparin and Celecoxib have been associated with a few side effects (Alban, 2012; Veettil et al., 2019), some of these side effects might in fact be beneficial for dystrophic muscles. For instance, Celecoxib was first developed and approved by the FDA for its anti-inflammatory effects. Moreover, over the years, Celecoxib has been examined for its potential to activate other targets. For example, it has recently been approved to be repurposed for the treatment of familial adenomatous polyps (Steinbach et al., 2000). Interestingly, a recent study revealed that Celecoxib attenuates oxidative stress in rats with colon cancer (Hegazi et al., 2017). Furthermore, Heparin, used clinically as an anticoagulant, was also shown to have anti-inflammatory properties in patients affected by asthma, inflammatory bowel disease, cardiopulmonary bypass and cataract surgery (Mousavi et al., 2015). In addition, Heparin has antioxidant effects in patients with end-stage renal disease (Nassiri et al., 2009). As mentioned previously, DMD patients suffer from an atypical increase of inflammatory macrophages in their muscles which provokes chronic inflammation, oxidative stress, necrosis and replacement of the muscle by connective tissue (Miyatake et al., 2016). Thus, Celecoxib and Heparin’s anti-inflammatory and antioxidative effects as well as their ability to promote utrophin A upregulation, while also improving muscle fiber integrity, may prove to have collective beneficial effects in DMD patient muscles.
5.6 Combinatorial Therapy to Induce an Additive Increase of Utrophin A

Increasing evidence suggest that drug combinations of two or more compounds could be a good way to increase the efficiency of treatments including compounds with low potency (Cordova et al., 2018; Ismail et al., 2018; Sun et al., 2016). Such combinatorial approach could consist of using different compounds to obtain a stronger increase/decrease of a desired molecule or to amplify a specific phenotype (Cordova et al., 2018; Ismail et al., 2018; Weiss and Nowak-Sliwinska, 2017). Moreover, an alternative combinatorial approach could comprise of using different therapies targeting genotype and phenotype such as gene therapy and pharmacological therapy (Ismail et al., 2018; Weiss and Nowak-Sliwinska, 2017). Combinatorial therapy is currently being explored in a DMD context, in order to target the pathological features such as muscle weakness, muscle wasting, degeneration, and inflammation (Cordova et al., 2018). For instance, a pilot clinical trial with L-arginine and metformin showed synergistic beneficial effects on muscular metabolism in DMD patients’ skeletal muscle tissues (Hafner et al., 2016). In the work presented here, we performed a 4-week combinatorial treatment with the utrophin A post-transcriptional activator Heparin and transcriptional activator AICAR. We demonstrated that the combination of both drugs activating utrophin A through distinct pathways, induced an additive effect on utrophin A expression levels in the TA, diaphragm and the heart. Interestingly, individual treatments of Heparin or AICAR did not increase utrophin A in the heart, however, a combination of both drugs induced a significant increase of utrophin A. This reveals the potential of combining different utrophin-activating drugs or small molecules to promote a synergistic effect in dystrophic muscles. Furthermore, a previous study treated mdx mice with two drugs (AICAR and GW501516) known to activate utrophin A through transcriptional events, and they demonstrated no additive effects on utrophin A expression levels compared to individual treatments (Jahnke et
al., 2012). This suggests that in order to have additional upregulation of utrophin A with a combinatorial treatment, it requires two compounds or more, that activates utrophin A through distinct signaling pathways. Thus, combinatorial treatment using drugs targeting distinct signaling pathways, may prove to be an efficient way to promote a higher increase of utrophin A in dystrophic muscles than individual treatments, and potentially induce a synergistic beneficial effect in DMD patients.

5.7 Targeting eEF1A2 with FDA-Approved Drugs to Promote IRES-Dependent Translation of Utrophin A

In our final manuscript (chapter 4), we described the potential of targeting eEF1A2, to promote IRES-dependent translation of utrophin A in skeletal muscles. First, we demonstrated that direct overexpression of eEF1A2 in wild-type, mdx and IRES-transgenic mice induced a striking increase of utrophin A protein levels and stimulated the activity of the 5’UTR IRES utrophin A reporter construct in skeletal muscles. This, in combination with a past study from our lab revealing eEF1A2’s ability to interact with utrophin A’s 5’UTR IRES site exclusively in skeletal muscles (Miura et al., 2010), suggests that eEF1A2 regulates utrophin A protein expression by activating IRES-dependent translation in vivo. These results demonstrated that targeting eEF1A2 with specific drugs would be a possible treatment to enhance expression of utrophin A in DMD muscles.

As described above, eEF1A2 plays a role in translation elongation where, when in its active GTP-bound form, it transports aminoacylated tRNAs to the ribosomal A site to permit protein synthesis (Ann et al., 1991; Browne and Proud, 2002; Knudsen et al., 1993b; Lund et al., 1996; Merrick, 1992; Moldave, 1985). Due to the multitude of eEF1A2 targets, the potential for off target effects could impact the feasibility of therapies aimed at modulating eEF1A2 levels. First, eEF1A2
has been shown to be upregulated in many types of cancers including breast, ovarian, pancreatic, prostate and lung cancers (Anand et al., 2001; Kawamura et al., 2014; Lee and Surh, 2009; Pinke et al., 2008; Sun et al., 2014; Veremieva et al., 2014; Worst et al., 2017; Zang et al., 2015). However, it is important to note that the involvement of eEF1A2 in cancer has only been shown in tissues where it is not typically expressed. In fact, eEF1A2 is expressed explicitly in skeletal muscle, heart and brain tissues, therefore, endogenous upregulation by pharmacological therapy should activate eEF1A2 specifically in these tissues (Knudsen et al., 1993; Lee et al., 1992). In addition, our lab has previously generated and characterized transgenic mice containing a bicistronic construct with the utrophin A IRES and observed that the reporter construct driven by a ubiquitous promoter, was strictly activated in skeletal muscles (TA and gastrocnemius) and was not detected in the lung, kidney, heart, liver or the brain. Furthermore, it is also highly unlikely that eEF1A2 would affect other mRNAs containing an IRES since the literature indicates that individual IRES have unique sequences and they appear to be regulated by distinct ITAFs (Baird et al., 2006). In fact, ITAFs mostly interact with its target mRNAs due to its specific localization and the IRES’ secondary structure (Pickering et al., 2004). Thus, activation of eEF1A2 by drug treatment will, in all likelihood, result in utrophin A-specific effects in skeletal muscle.

By performing a high-throughput FDA-approved drug screen, we identified 11 drugs that increased both eEF1A2 and utrophin A protein levels in skeletal muscle cells. These FDA-approved drugs had common roles and could be separated in seven classes including anti-diabetic, anti-peptic ulcer, cholesterol-lowering and beta-adrenergic blocking agents, which demonstrates the authenticity of our approach. Two of these drugs, Betaxolol and Pravastatin activated a bicistronic reporter construct containing utrophin A’s 5’UTR IRES site in vitro and in vivo in our
transgenic mice. Moreover, we observed a significant improvement in the dystrophic phenotype of mdx mice, following a 4-week treatment with each drug.

In order to determine that utrophin A protein upregulation following treatment was dependent on eEF1A2 activity, we treated wasted mice (eEF1A2 null mice) with Pravastatin. These wasted mice are characterized by decreased body weight (likely due to muscle wasting), neurological and immunological irregularities starting at 21 days after birth resulting in their death at 28 days (Abbott et al., 2009; Chambers et al., 1998). In wild-type neuronal, cardiac and skeletal tissues, eEF1A1 is highly expressed throughout embryonic development, gradually declines after birth and is undetectable by day 21. Whereas, eEF1A2 expression in these tissues rises starting shortly before birth until it reaches a plateau at day 21 (Khalyfa et al., 2001). Therefore, we treated wild-type and wasted mice with Pravastatin starting at day 20 for 5 days. Our results revealed that the drug treatment induced a significant increase of utrophin A in wild-type mice but not in the wasted mice, thus, revealing the dependency of utrophin A regulation by Pravastatin on eEF1a2.

These data show the advantages of targeting eEF1A2 in dystrophic muscles to induce IRES-dependent translation and shows a new approach to identify FDA-approved drugs for the treatment of DMD.

5.8 Betaxolol and Pravastatin as Potential Treatments for DMD

In chapter 4, we identified Betaxolol and Pravastatin as activators of eEF1A2 and showed their ability to promote IRES-dependent translation of utrophin A in skeletal muscle. In addition, a 4-week treatment with these drugs induced a significant improvement of the dystrophic phenotype of mdx mice. In fact, both drugs had a striking effect on muscle fiber integrity, as shown by the strong decrease of IgM penetration in the cytoplasm of dystrophic muscle fibers. These
results correlated with the amplified recruitment of utrophin A at the sarcolemma muscle fibers treated with either Betaxolol or Pravastatin compared to vehicle control, which provides support and flexibility during muscle contractions. In addition, both drugs improved forelimb and hindlimb muscle grip strength in mdx mice, which reveals functional improvements in mdx mice. The two drugs significantly reduced the number of centrally nucleated muscle fibers, demonstrating their ability to decrease muscle damage and regenerating fibers (Briguet et al., 2004). Collectively, these findings demonstrate the beneficial effects of Betaxolol and Pravastatin in dystrophic muscles and highlight their potential as therapies for DMD.

Betaxolol and Pravastatin were originally developed for other functions. Betaxolol is currently used as a beta-adrenergic blocking agent to treat hypertension and Pravastatin is typically used as a cholesterol lowering drug. The beta-adrenergic blocker Carvedilol, was reported to ameliorate heart function in DMD patients (Rhodes et al., 2008). Taking these functions into account with our study, Betaxolol may have dual beneficial effects in DMD patients by 1) increasing utrophin A and therefore providing strength and integrity to the dystrophic muscles in combination with 2) improving cardiac function, which is important since cardiac dysfunction is one of the main causes of death in DMD patients. Moreover, a recent study reports that the statin, simvastatin reduces oxidative stress markers in mdx mice (Whitehead et al., 2016), thus, Pravastatin may have antioxidant properties that could improve the dystrophic phenotype of DMD patients alongside the beneficial effects by utrophin A upregulation in dystrophic muscles. Altogether, FDA-approved drugs Betaxolol and Pravastatin are multifunctional drugs that may potentiate a synergistic effect in DMD.
5.9 Drug Repurposing as a Therapeutic Approach to Treat DMD

Many therapies are being developed to find a cure for DMD such as gene therapy (Harper et al., 2002; Kodippili et al., 2018; Wang et al., 2012; Wasala et al., 2018), cell therapy (Benedetti et al., 2013; Cossu et al., 2015; Crist, 2017; Danisovic et al., 2018), gene editing (Amoasii et al., 2018; Bengtsson et al., 2017; Duchêne et al., 2018; Hakim et al., 2018; Ifuku et al., 2018; Long et al., 2014; Nelson et al., 2016; Xu et al., 2018), and more; however, pharmacological therapy with glucocorticoids is the only current therapy being used today (Birnkrant et al., 2018a). Pharmacological treatment has many benefits over gene and cell-based therapies including little to no toxicological concerns or undesired immune responses as well as practical systemic administration and ease of delivery to the muscles (Guiraud and Davies, 2017). Yet, one disadvantage of drug therapy is that it may modulate alternative signaling molecules other than the desired targeted pathway, which could lead to unwanted side-effects (Zhang et al., 2013). Therefore, additional studies are usually required to determine these secondary effects.

An alternate method such as drug repurposing, does not present the same risks as a new drug or small molecule development. As described above, drug repurposing consists of using FDA-approved or investigational drugs outside their original medical function or initial target disorder (Ashburn and Thor, 2004). In the past years, researchers have identified FDA-approved drugs to use in a DMD context such as Nabumetone (Moorwood et al., 2011), Safinamide (Vitiello et al., 2018), Tamoxifen (Wu et al., 2018), Simvastatin (Whitehead et al., 2015, 2016) as well as many different flavanols such as quercetin (Selsby et al., 2016). Previous studies from our laboratory have also exploited the off-target effects of many repurposed drugs that were found to stimulate utrophin A in muscle including Resveratrol, Metformin, AICAR (Al-Rewashdy et
al., 2015; Ljubicic and Jasmin, 2015; Ljubicic et al., 2011, 2014; Miura et al., 2009), along with Heparin, Celecoxib, Betaxolol and Pravastatin characterized in this work.

This approach has numerous benefits compared to novel drug development (Pushpakom et al., 2019) including: 1) Toxic dose analysis and off-target effects have been determined in various animal models as well as in humans. Thus, the repurposed drug is less likely to fail safety assessment in clinical trials; 2) this results in faster drug development times as well as 3) reduced costs in pre-clinical and early clinical testing. In fact, the average costs of repositioning a drug in the US and translating it to marketplace is approximately $300 million compared to ~$2.5 billion for a new drug (Nosengo, 2016). A few barriers make the process of drug repurposing difficult such as patents and the lack of partnership especially between academia and industry. However, a few important collaborations are emerging between pharmaceutical companies, small industries and academia. For example: The AstraZeneca Open Innovation Platform, The Center for Excellence for External Drug Discovery (GlaxoSmithKline) as well as Centers for Therapeutic Innovation at Pfizer, all capitalize on collaborations to increase accessibility to well-characterized compounds suitable for drug repurposing (Pushpakom et al., 2019). In addition, an increasing number of partnerships are being formed between patient advocacy groups, charities and academia (such as Cures Within Reach - https://www.cureswithinreach.org) to repurpose generic drugs towards rare diseases. Overall, repurposing drugs is an emerging field that could permit the quick and safe development of therapies for rare diseases such as DMD.
5.10 Future Directions and Conclusions

In this work, we repurposed four promising FDA-approved drugs that were shown to upregulate utrophin A expression levels in dystrophic muscles: Heparin, Celecoxib, Betaxolol and Pravastatin. In chapter 2 and 3, we revealed that a long-term (4-week) treatment with Heparin and Celecoxib increased utrophin A expression levels through the activation of p38 MAPK which resulted in significant improvements in mdx mice muscles. Future directions would consist of determining if TLRs are involved in the activation utrophin A upstream of p38 MAPK. To do so, TLR expression levels would need to be assessed in skeletal muscle cells and mdx muscles pre- and post-Heparin or -Celecoxib treatment. Next, siRNAs targeted towards the individual TLRs could be used to determine if they inhibit utrophin A upregulation by the drug treatments in skeletal muscle cells. In parallel, treatment of muscle cells with specific ligands, such as lipopolysaccharide (LPS), known to interact with and activate TLRs, could be used to determine if it promotes an increase in utrophin A. Results from these experiments would provide valuable information geared towards understanding the mechanisms by which TLRs are activated via Heparin or Celecoxib and if these TLRs activate utrophin A through p38 MAPK dependent signaling. In addition, since p38 MAPK isoforms seem to have contradicting effects in muscles (Pogozelski et al., 2009; Wissing et al., 2014), future directions would involve using distinct p38 MAPK antibodies to determine which p38 isoform is activated in Heparin or Celecoxib treated mdx mice muscles.

In this study we also describe the potential of combinatorial treatment with drugs that activate utrophin A through distinct pathways and their additive effect on utrophin A upregulation. It would be interesting to further investigate combinatorial treatment in mdx mice with a p38-activating drug (Heparin or Celecoxib) and an eEF1A2-activating drug (Betaxolol...
or Pravastatin) to see if they have an additive effect on utrophin A expression but also promote synergistic beneficial effects in dystrophic muscles. Furthermore, future work to perform a combinatorial treatment with Celecoxib and glucocorticoids would also be of great interest since it has been shown that inhibition of COX-2 by Celecoxib enhances glucocorticoid receptor function in neuronal PC12 cells (Hu et al., 2005). Thus, a combinatorial treatment with glucocorticoids and Celecoxib may have either additive or even synergistic effects in patients with DMD and could be seamlessly incorporated in treatment plans since the majority of DMD patients are undergoing glucocorticoid treatment.

Finally, our last study (chapter 4) can be separated in two main findings: 1) using wild-type, mdx and transgenic mouse models, we showed that eEF1A2 directly regulates endogenous protein expression of utrophin A via IRES-dependent translation in vivo. 2) We developed a successful high-throughput screening strategy targeting eEF1A2 and identified 11 FDA-approved drugs able to activate cap-independent translation of utrophin A in vitro. Further investigation of two of these drugs, Betaxolol and Pravastatin, revealed their ability to stimulate this pathway in vivo as well as strikingly, ameliorating the dystrophic phenotype of mdx mice. These novel findings gave rise to many possibilities of future directions including investigating the effects of the 9 remaining drugs identified in the high-throughput drug screen on the dystrophic phenotype in mdx mice as well as determining the direct effect of upregulating eEF1A2 in mdx mice. To do so, we would crossbreed eEF1A2 expressing founder mice with mdx mice, to study the extent of the dystrophic pathology of these transgenic mdx mice at different ages. This would demonstrate if eEF1A2 overexpression induce beneficial effects in mdx mice. As a control experiment we would crossbreed eEF1A2-null mice with mdx mice to determine if the dystrophic phenotype worsens.
Considering that our high-throughput drug screen contained only 262 drugs, other future perspectives would be to use other large-scale FDA-approved drug libraries that are available (for example: Screen-Well from Enzo-Life Sciences, DiscoveryProbe from APExBIO, Prestwick from Prestwick Chemicals and LOPAC from SIGMA-ALDRICH) to identify additional compounds that could target eEF1A2 and utrophin A. Lastly, we would aim to bring our best potential FDA-approved drugs to clinical trials.

In conclusion, DMD is a devastating disease that is complex to treat for two main reasons: 1) mutations found in the gene encoding dystrophin of DMD patients vary from one person to another, affecting different areas of the gene and ranging from a single-exon deletion/duplication to multi-exon mutations; 2) dystrophin plays a central role in muscle, thus, the lack of dystrophin results in a cascade of unwanted side-effects which significantly increase the severity of the disorder. In this work, we propose a safe treatment method, using specific FDA-approved drugs to upregulate utrophin A to compensate for the loss of dystrophin, which is a therapy applicable to each DMD patient and that can collectively treat all affected muscles. We demonstrate that treatment with these drugs upregulates utrophin A resulting in significant improvement of the dystrophic phenotype, but also may produce additional beneficial effects by ameliorating hallmark features of DMD such as inflammation and oxidative stress. Overall, drug repurposing is an emerging technique that results in faster development times, reduces health risks and lowers costs, therefore, a therapy using FDA-approved drugs aimed at upregulating utrophin A, is a promising therapeutic strategy for DMD.
References


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Summit Therapeutics plc (2018). Ezutromid significantly reduced muscle damage in DMD patients in 24-week interim - data from Summit’s PhaseOut DMD clinical trial.


Appendix A
CHRISTINE PÉLADEAU

EDUCATION:

Ph.D. in Cellular and Molecular Medicine  
University of Ottawa, Ottawa, Ontario  
2015 – 2019

M.Sc. in Cellular and Molecular Medicine  
University of Ottawa, Ottawa, Ontario  
2010 – 2013

Applied B.Sc. in technology – Biotechnology – COOP  
La Cité Collégiale, Ottawa, Ontario  
2005 – 2009

WORK EXPERIENCE:

Ph.D. in Cellular and Molecular Medicine  
University of Ottawa, Ottawa, Ontario  
January 2015 – May 2019

Supervisor: Dr. Bernard Jasmin, Dean of the faculty of Medicine

Objective: Use new technologies to perform a chemical screen to identify novel drugs or small molecules that may be beneficial for treatment of Duchenne Muscular Dystrophy.

- Co-Supervised an undergraduate honours student, co-op student and a laboratory technician
- Performed high-throughput screen of drugs of interest via ELISA
- Ran PCR, RT-PCR and qPCR analyses of mRNA target molecules
- Used a cryostat to generate tissue cross-sections and performed downstream immunofluorescence staining
- Performed muscle tissue protein extractions and downstream immunoblotting
- Wide array of animal work with mice including behavioural testing, injections, and dissections

Ph.D. internship – Policy Analyst  
May 2018 – September 2018

National Research Council Canada, Stakeholder Relations and Parliamentary Affairs (SRPA)

Supervisor: John Burnett

- Research, collect and analyze information to create lay summaries for the President
- Create meeting notes and briefing notes for the President
- Working collaboratively with internal or external stakeholders

Laboratory technician 1  
June 2013 – December 2014

University of Ottawa, Ottawa, Ontario

Supervisor: Dr. Bernard Jasmin

- Worked with mice including transgenic colony maintenance and associated genotyping
- Performed muscle tissue protein extractions and accompanying immunoblotting
- Ran radioactive PCR, RT-PCR, QPCR
- Assessed protein localization by immunofluorescence
• Responsible for laboratory maintenance and inventory
• Trained an undergraduate student

**M.Sc. in Cellular and Molecular Medicine**  
*September 2010 – June 2013*  
University of Ottawa, Ottawa, Ontario  
Supervisor: Dr. John Copeland

**Objective:** Determine if a specific isoform of Formin-like 2 is essential for amoeboid style invasion as well as to determine the effect of alternative splicing of FMNL2 on the regulation and activity of the encoded proteins

• Performed PCR, RT-PCR, QPCR and Immunoblotting
• Transfected cancer cells with siRNA’s to knockdown specific isoforms
• Used a confocal microscope and immunofluorescent techniques to visualize cancer cell movement
• Performed cell based assays: invasion, migration and wound healing
• Experienced in sterile tissue culture procedures
• Trained undergraduate students

**Laboratory Technician**  
*April 2009 – May 2010*  
National Research Council Canada (NRC-IBS), Immunomodulation / VBI Vaccines, Ottawa, Ontario  
Supervisor: Dr. Dennis Sprott

• Optimized media culture content for ideal Archea bacteria growth in bioreactors
• Extracted phospholipids from bacteria to create liposomes
• Purified lipids by column chromatography
• Used mass spectrometry MALDI system to identify the lipids in our samples
• Performed ELISA assays

**Junior Laboratory Technician – COOP and Honour’s project**  
*May 2008 – August 2008*  
National Research Council Canada (NRC-IBS), Ottawa, Ontario  
Supervisor: Dr. Dennis Whitfield

• Synthesized lipopeptides and glycopeptides using organic chemical techniques and a specialized microwave
• Purified our product by HPLC
• Identified our product by NMR 400 analysis

**Junior Laboratory Technician in organic chemistry – COOP**  
*April 2007 – May 2008*  
Paracel Laboratories Ottawa, Ontario

• Performed petroleum hydrocarbons extractions
• Prepared solvents
• Responsible for laboratory maintenance and ordered laboratory supplies
SKILLS:

- **Laboratory experience:**
  - Biotechnology, Molecular biology, Microbiology, bioproduction, industrial microbiology, industrial biochemistry, instrumentation I and II, bioinformatics I and II, organic chemistry, analytical chemistry, general chemistry, cell biology, tissue culture,
  - Sound knowledge of laboratory maintenance and safety practices

- **Instruments:**
  - Chromatography: EC, GC, HPLC
  - Detectors: FID, MS, UV-Vis
  - Microscopy: Fluorescent, Confocal, Spinning Disk, EVO cell imaging system
  - Spectroscopy: Atomic absorbance, Fluorescence, UV-Vis
  - Others: Autoclave, Bioreactor, MS MALDI, NMR 400, PCR, RT-PCR, QPCR, rotary evaporators

Strongly proficient in Microsoft Word, Excel, Power Point, Adobe Illustrator and Photoshop

- **Team work:**
  - Working in a group setting to carry out analyses and to solve problems.

- **Management:**
  - Self-motivated and excel in meeting deadlines
  - Co-supervised and mentored undergraduate students and a junior lab technician.

PUBLICATIONS:

- Pélaudeau C *et al.* Identification of novel therapeutics that target eEF1A2 and upregulate utrophin A translation in dystrophic muscles (Submitted to Nature Communications – April 3rd, 2019 – sent to reviewers).
- Pélaudeau C *et al.* Celecoxib treatment improves muscle function in mdx mice and increases utrophin A expression. FASEB. 2018, 32(9), 5090-5103.
- Pélaudeau C *et al.* A Specific FMNL2 Isoform is Up-Regulated in Invasive Cells. BMC Cell Biol. 2016, 17, 1-12

ADDITIONAL INFORMATION:

- **Oral presentations - Invited speaker:**
  - Event: CMM/Neuroscience Research day, University of Ottawa, Ottawa, Canada  November 2017
  - Title: Targeting eEF1A2 with FDA-approved drugs to upregulate utrophin A translation in dystrophic muscles
• Event: Seminar course 4th year cohort, La Cité Collégiale, Ottawa, Canada  March 2016
Title: Recherche et développement de thérapies; Mon cheminement de l’industrie aux études supérieures.

➢ Poster presentations:
• 4th Ottawa International Conference on NMD and Biology, Ottawa, Canada 2017
• CIHR Research Poster Presentation.- Canadian Student Health Research Forum, Winnipeg 2017
  *nominated as being within the top 5% of doctoral students in Canada - Award: silver award (250$)
• 3rd Ottawa international conference on NMD and Biology, Ottawa, Canada 2015
• RNA society, Québec, Canada 2014
• American Society For Cell Biology, Philadelphia and Denver, USA 2010, 2011

➢ Recognition Awards:
• Ontario Graduate Scholarship (OGS) (Province of Ontario) (15000$) 2018-2019
• Excellence Scholarship (University of Ottawa) (10000$) 2018-2019
• CNMD Scholarships in Translational Research (STaR) award - (10000$) (declined) 2018-2019
• CIHR poster pres. silver award, Canadian Student Health Research Forum (250$) 2017
• CIHR travel grant for Canadian Student Health Research Forum (1000$) 2017
• CNMD Scholarships in Translational Research (STaR) award – (10000$) 2017-2018
• CNMD Scholarships in Translational Research (STaR) award – (10000$) 2016-2017
• Admission scholarship for an average over 8.0 (University of Ottawa) (36000$) 2015-2019
• The University of Ottawa Dean's scholarship (1000$) 2013
• Admission scholarship for an average over 8.0 (University of Ottawa) (15000$) 2010-2012
• Scholarship for an average of over 3.2 / 4.3 (3000$) 2007-2009